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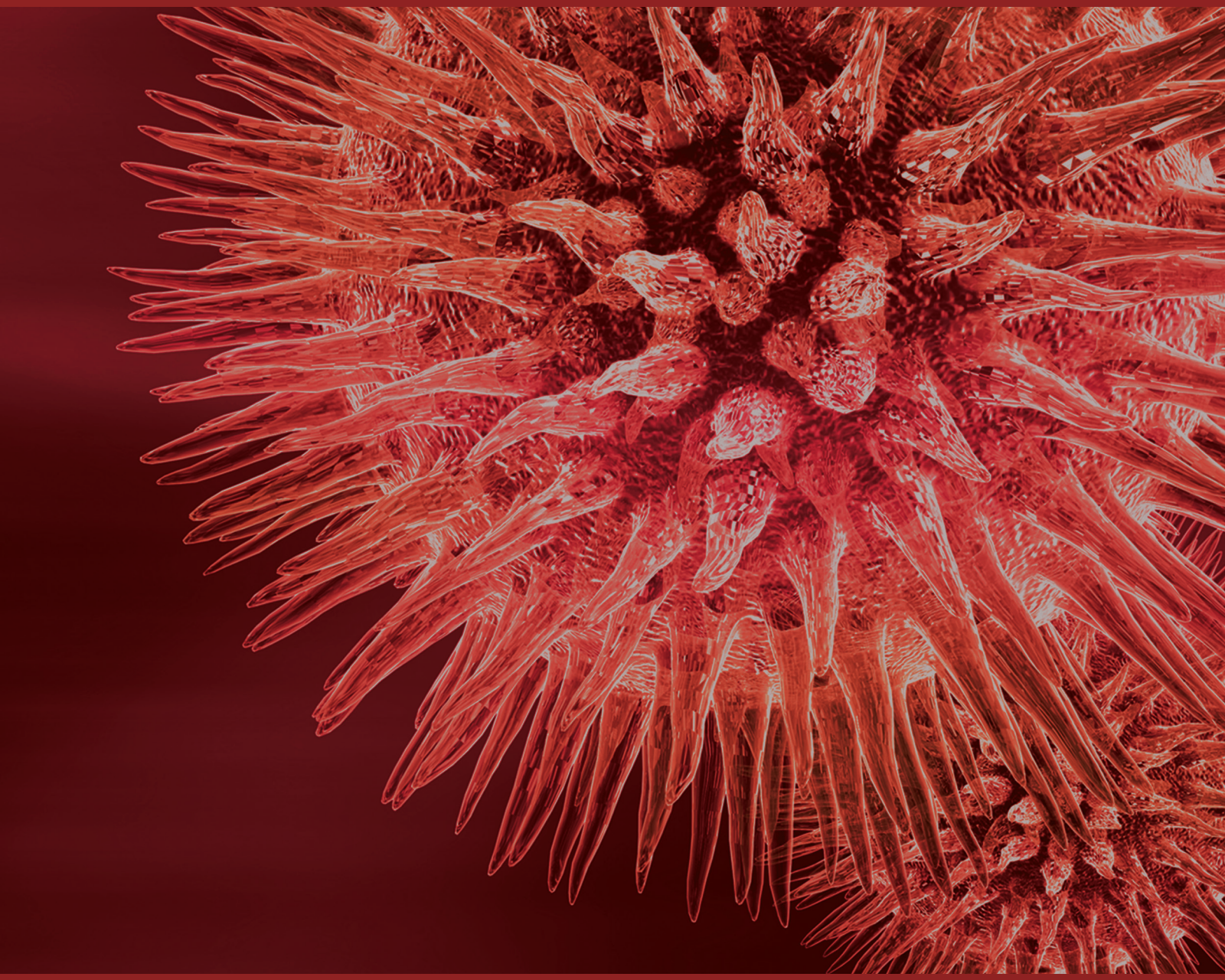
Epidemiology, Detection, and Control of Foodborne Microbial Pathogens

Guest Editors: Miguel Prieto, Pierre Colin, Pablo Fernández-Escámez, and Avelino Alvarez-Ordóñez



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Editorial

Epidemiology, Detection, and Control of Foodborne Microbial Pathogens

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Foodborne microbial diseases are a significant public health threat. They occur in both developed and developing countries with different food industry expansion, food safety regulations, food hygiene and consumption habits, and climate and environmental situations. The subsequent economic burden associated to them is also different. Most foodborne diseases are sporadic and often not reported, but sometimes foodborne outbreaks may affect a large number of individuals and compromise economic sectors and sanitary resources. Contamination of foods can occur at any point along the food chain, while pathogenic microorganisms enter the body through the ingestion of contaminated food.

A series of pathogens and diseases are emerging, driven by factors such as the change in pre- and postharvest stages of food production and manufacturing, pathogen adaptation and evolution, and changes in lifestyle, consumption habits, or host susceptibility, which modify the global exposure to foodborne pathogens. Other factors contemplated as possible contributors to the increased incidence include the recent changes in the food supply system, which result in intensive production and complexity of the supply chain. Because food production, manufacturing, and marketing are global, infectious agents present in foods can be disseminated from the original point of processing and packaging to the place of consumption thousands of kilometers away. Travel and expansion in international trade of foods have also increased the occurrence of outbreaks involving several countries and of cross-border transmission of agents and diseases.

There are a number of recent advances in the epidemiology, detection, and control of foodborne agents, including the development of novel methodologies and tools for the detection of pathogens, the growing availability of genome sequences which provide unrestricted information on bacterial genetics and physiology and give clues for the control of pathogenic microorganisms, the expanding knowledge on the epidemiology of the emerging pathogens, and the extensive application of molecular genomics and postgenomics tools for deciphering the behavior of foodborne pathogens in food-related environments. Surveillance studies contribute also to providing data and a better understanding into the existence and spread of foodborne pathogens. There is a special interest to know the origin and routes of contamination of foodborne pathogens, while farm animals and domestic and wild animals have been reported to be primary reservoirs for foodborne pathogens. It is also increasingly known that foodborne pathogens are able to change and adapt to different environmental conditions. The ability of these pathogens to develop adaptive response networks has contributed to their capacity to survive under a wide range of conditions and even stimulate their virulence potential.

This special issue comprises several reviews and original research articles which cover the most recent investigations on aspects such as the occurrence of foodborne pathogens, molecular typing of isolates, methods of detection or strain characterization aimed at foodborne pathogens, investigation and management of outbreaks, mathematical modelling of

microbial growth and inactivation in foods, novel technologies of food preservation and microbial stress responses, and the impact of all this knowledge on food safety management, including the design of novel strategies for the (bio)control of foodborne pathogens.

Regarding occurrence surveys and molecular epidemiology studies, the contributions address a variety of relevant foodborne pathogens at international level in different animal and human populations and food categories (*Campylobacter jejuni* in poultry in Italy by F. Marotta et al.; Shiga toxigenic *Escherichia coli* O157 and non-O157 in ruminant feces in Malaysia by A. Perera et al.; *Salmonella enterica* in poultry and humans in Algeria by R. Elgroud et al.; enteropathogenic *Yersinia* spp. in pigs in Finland by M. J. Vilar et al.; food- and smear-transmitted pathogens [*E. coli*, norovirus, *Shigella* spp., *Cryptosporidium parvum*, *Giardia duodenalis*, *Salmonella* spp., astrovirus, *Rotavirus*, and *Sapovirus*] in humans in Mali by H. Frickmann et al.; *Listeria ivanovii* in ready-to-eat foods and food processing environments in Ireland by A. Alvarez-Ordóñez et al.; *Listeria* spp. in food samples in Brazil by D. C. Vallim et al.; *Arcobacter butzleri* and *Arcobacter cryaerophilus* in pork, beef, and chicken meat in Poland by I. Zacharow et al.; *Escherichia coli* in conventional and free-range poultry in Brazil by V. L. Koga et al.; *Listeria monocytogenes* in hospital patients in Spain by J. Ariza-Miguel et al.).

A number of studies deal with methods of detection and isolation of foodborne pathogens and characterization of isolates. M. Srisawat and W. Panbangred describe a loop-mediated isothermal amplification method (LAMP) for the specific detection of *Salmonella* in food samples. A. Rohde et al. perform a systematic comparison of different homogenization approaches (stomaching, sonication, and milling) for the accurate detection of foodborne pathogens in meat. K. Jaakkola et al. report a comparative genomic hybridization analysis with a DNA microarray based on three *Yersinia enterocolitica* and four *Yersinia pseudotuberculosis* genomes to shed light on the genomic differences between enteropathogenic *Yersinia*. One research article by F. Sayk et al. describes the management activities implemented by the Emergency Department in relation to the German Shiga Toxin producing *Escherichia coli* (STEC) O104:H4 outbreak in two tertiary hospitals in Lubeck, northern Germany, in relation to patients with food-related diarrhoea.

Two studies are related to microbial growth in foods. C. M. McAuley et al. evaluate the growth of *Salmonella* Typhimurium and *Salmonella* Sofia on eggs under conventional production and retail conditions. M.-L. Pla et al. compare the accuracy of a variety of primary models to predict the growth of *Bacillus cereus*, *Listeria monocytogenes,* and *Escherichia coli* by the plate count and absorbance methods.

Several contributions focus on novel food preservation methodologies and food control strategies and on how microbial stress responses impact their efficacy. L. Wang and C. Shen assess the efficacy of hops beta acids (HBA) against unstressed and stress-adapted *Listeria monocytogenes* in ham extract and evaluate the consumers' acceptability of HBA on ready-to-eat ham. M. Gouma et al. establish the process criteria for using UV-C light alone or combined with mild heat (UV-H treatment) to inactivate 5 $\log_{10}$cycles of *Escherichia coli*, *Salmonella* Typhimurium, *Listeria monocytogenes,* and *Staphylococcus aureus* in chicken broth. T. Hintz et al. review the use of plant-derived products as antimicrobial agents for food preservation to control foodborne pathogens. L. Guevara et al. assess the impact of moderate heat, combined with carvacrol and thymol on the viability, injury status, and stress response of *Listeria monocytogenes*. E. Jończyk-Matysiak et al. review the potential as control strategies of bacteriophages active against *Bacillus anthracis* and other *Bacillus cereus* group members.

This editorial summarizes the topics discussed in the articles published in this special issue, in the confidence that readers will find this information useful with the most recent research on major developments in the area of epidemiology, detection, and control of foodborne microbial pathogens.

Acknowledgments

We would like to thank the authors and reviewers for their valuable contributions and constructive criticisms to this special issue. We sincerely hope that this collection of papers will prompt further research and contribute to novel or improved strategies of food safety management to be able to further reduce the incidence of foodborne microbial diseases.

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Research Article

Comparative Genomic Hybridization Analysis of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* Identifies Genetic Traits to Elucidate Their Different Ecologies

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Enteropathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* are both etiological agents for intestinal infection known as yersiniosis, but their epidemiology and ecology bear many differences. Swine are the only known reservoir for *Y. enterocolitica* 4/O:3 strains, which are the most common cause of human disease, while *Y. pseudotuberculosis* has been isolated from a variety of sources, including vegetables and wild animals. Infections caused by *Y. enterocolitica* mainly originate from swine, but fresh produce has been the source for widespread *Y. pseudotuberculosis* outbreaks within recent decades. A comparative genomic hybridization analysis with a DNA microarray based on three *Yersinia enterocolitica* and four *Yersinia pseudotuberculosis* genomes was conducted to shed light on the genomic differences between enteropathogenic *Yersinia*. The hybridization results identified *Y. pseudotuberculosis* strains to carry operons linked with the uptake and utilization of substances not found in living animal tissues but present in soil, plants, and rotting flesh. *Y. pseudotuberculosis* also harbors a selection of type VI secretion systems targeting other bacteria and eukaryotic cells. These genetic traits are not found in *Y. enterocolitica*, and it appears that while *Y. pseudotuberculosis* has many tools beneficial for survival in varied environments, the *Y. enterocolitica* genome is more streamlined and adapted to their preferred animal reservoir.

1. Introduction

Enteropathogenic *Yersinia* is the third most common cause of bacterial enteritis in European countries, even though a statistically significant decreasing 5-year trend in yersiniosis cases has been reported in the EU [1]. Infection is usually foodborne, with symptoms ranging from self-limiting diarrhea to reactive arthritis or erythema nodosum [2]. *Yersinia* are Gram-negative rods belonging to Enterobacteriaceae. Enteropathogenic *Yersinia* diverged around 41–185 million years ago, while the third human pathogenic species of *Yersinia* genus, the infamous *Yersinia pestis*, is a relatively recent clone of *Yersinia pseudotuberculosis* [3]. The evolution of enteropathogenic *Yersinia* is thought to have included multiple distinct ecological specializations that have separated the pathogenic strains from environmental, nonpathogenic lineages. This current hypothesis of parallel evolution [4] rejects the previous one suggesting that all pathogenic *Yersinia* species share a common pathogenic ancestor [5].

Enteropathogenic *Yersinia enterocolitica* and *Y. pseudotuberculosis* cause similar infections, but their epidemiology and ecology appear to differ in many aspects. Both *Y. enterocolitica* and *Y. pseudotuberculosis* have been isolated from swine or pork, and yersiniosis has been associated with the consumption of uncooked pork [6–8]. Traditionally, most cases of yersiniosis are thought to occur sporadically, and cases caused by *Y. enterocolitica* are mostly associated with pork products [7, 9–11]. In rare cases, the source of human infection has been traced back, for example, to milk, poultry meat, and ready-to-eat salad [12–14]. Within recent decades, several widespread outbreaks caused by *Y. pseudotuberculosis* have been reported in Finland [15–18]. The sources of the infections have been traced back to fresh produce, such as iceberg lettuce [15] and grated carrots [18–20]. The epidemic

strain involved in an outbreak caused by raw carrots was also recovered from the field and production line [19]. The genetic traits underlying the observed epidemiological differences remain poorly understood.

Research has shown that the prevalence of *Y. enterocolitica* in swine is notably higher than that of *Y. pseudotuberculosis*, and swine are the only reservoir from which *Y. enterocolitica* 4/O:3 strains have regularly been isolated [2, 25–27]. The most common cause of *Y. enterocolitica* infection in humans in Africa, Europe, Japan, and Canada is *Y. enterocolitica* 4/O:3. Bioserotype 4/O:3 is considered as an emerging pathogen, while the prevalence of the second most common pathogenic bioserotype, *Y. enterocolitica* 1B/O:8, is diminishing [2, 10, 28, 29]. Extensive research has been carried out to uncover the virulence factors of *Y. enterocolitica* and its different serotypes [30–35], and the virulence factors explaining the swine specificity of *Y. enterocolitica* serotype O:3 were recently identified [22, 36]. The differences in virulence gene expression patterns alter the surface adhesion properties and cytokine production profiles of O:3 strains and thus probably permit the asymptomatic infection and long-term colonization of the nasopharynx and intestine of swine [22].

Pathogenic and nonpathogenic *Y. enterocolitica* strains are frequently found from wildlife samples such as water fowl and hares, but pathogenic strains have rarely been isolated from soil or water [2, 8, 37]. *Y. pseudotuberculosis* strains have been isolated from a variety of sources, including fresh vegetables and wild animals, and contrary to *Y. enterocolitica*, all strains are considered pathogenic [8, 15–17, 38]. Despite the frequent presence of *Y. pseudotuberculosis* in environmental samples, its reservoir is considered to be wildlife [38, 39].

A comparative genomic hybridization (CGH) analysis with a DNA microarray based on three *Y. enterocolitica* and four *Y. pseudotuberculosis* genomes was conducted to shed light on the genetic traits and ecological specializations explaining the epidemiological differences between enteropathogenic *Yersinia*. Our hypothesis was that the genomes would contain operons elucidating the ways in which *Y. enterocolitica* has adapted to its mammal hosts and the ecology of *Y. pseudotuberculosis*. The strains hybridized on the microarray were isolated from human, animal, and environmental samples.

The hybridization results revealed that *Y. pseudotuberculosis* strainscarry many operons linked with the uptake of carbohydrates and use of aromatic substances that are absent from *Y. enterocolitica*. Phenolic compounds, polyamines, myoinositol, and aliphatic sulfonates are all substrates that are not commonly present in living animal tissue, but more abundant in plants and the soil environment. *Y. pseudotuberculosis* also harbors an array of different types of type VI secretion systems (T6SSs), in contrast to just one found in the *Y. enterocolitica* genome. These T6SSs are likely to provide defense against other bacteria and single-celled organisms in the environment.

2. Materials and Methods

2.1. Bacterial Strains for Hybridization. A total of 60 *Y. enterocolitica* and 38 *Y. pseudotuberculosis* strains isolated from a variety of geographic locations and sources were used in this study (Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/760494). The strains were selected to represent the different biotypes and serotypes of enteropathogenic *Yersinia*, and also included were three strains (*Y. enterocolitica* subsp. *enterocolitica* 8081, *Y. enterocolitica* subsp. *palearctica* Y11 [DSM 13030], and *Y. pseudotuberculosis* IP32953) used in the microarray design. These three strains were used as reference strains and as positive hybridization controls. The reference strains and one additional strain were hybridized in quadruplicate to assess the reproducibility of the hybridizations. The reference strains produced positive hybridization signals with 99.4–99.9% of the probes designed to hybridize with their sequences.

In total, 41 strains represented the most common pathogenic *Y. enterocolitica* bioserotype 4/O:3. The majority (79/98) of the strains had been isolated from swine or from swine slaughterhouses. The rest of the strains ($n = 19$) were isolated from human patients, wild birds, and other animals.

2.2. DNA Microarrays. The DNA microarrays were designed based on seven genomes and 14 plasmid sequences (Table S2) obtained from the NCBI database. 29,786 sequences were clustered into 11,564 gene groups by Cd-hit-est [41]. The threshold value of identity was set to 95% with minimum alignment of at least 80% of the longer sequence. Stringent clustering parameters were chosen to avoid problems with uncomplimentary probes in the probe design. With these parameters, the number of unique sequences (gene groups containing a sole sequence) amounted to 3747.

One 45–60-mer probe was designed for each gene group ($n = 11{,}564$). Thirteen gene groups containing a total of 14 sequences were excluded from the probe design because of redundancy. The longest gene sequences were over 10,000 bases long ($n = 11$), and for these a tiling method was used for the design of extra probes (10 per sequence). All probes were designed using Agilent Technologies Gene Expression Probe Design. Each of the eight subarrays of Agilent $8 * 15$ K custom arrays (Agilent, Santa Clara, CA, USA) contained an equal set of 11,661 probes.

2.3. Hybridization and Washes. Genomic DNA was isolated using a method described by Pitcher et al. [42]. A total of 500 ng of genomic DNA from each *Yersinia* strain was fluorescently labeled with the BioPrime ArrayCGH labeling module (Invitrogen, Carlsbad, CA, USA) using either Cy3 or Cy5 (GE Healthcare, Buckinghamshire, UK). For each hybridization, one Cy3-labeled and one Cy5-labeled DNA sample were combined. The mixture was purified with a DNA purification kit (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of DNA and the incorporation of the dye were checked with the Nanodrop device (Nanodrop Technologies, Wilmington, MA, USA) before and after labeling. The differently labeled DNA sample pairs to be hybridized into one of the eight subarrays on each array slide were randomly selected.

A volume of 2.2 μL salmon sperm DNA (1 mg/mL) was added to 17.8 μL of labeled combined sample solution, and the

mixture was heated at 95°C for 2 minutes for denaturation. A volume of 5 μL of 10x blocking agent (Agilent) and 25 μL of 2xGE (HI-RPI) hybridization buffer (Agilent) was added. A total of 45 μL of the solution was hybridized on each microarray at 65°C for 16 hours. The arrays were washed twice for 1 minute with Wash Buffer 1 (Agilent) and then for 1 minute with prewarmed Wash Buffer 2 (Agilent).

2.4. Scanning and Data Analysis. The CGH data analysis followed the routines set by Lindström et al. [43] and Lahti et al. [44]. The slides were scanned (Axon Genepix Autoloader 4200 AL, Molecular devices Inc., Sunnyvale, California, USA) with a resolution of 5 μm. Images were processed and manually checked using GenePix Pro 6.0/6.1 software. For data analysis, R software and the LIMMA package were used [40, 45]. For background correction, the normexp algorithm (offset 50) was applied [46].

The distribution of logarithmic signal intensities formed two clear peaks in all hybridizations and a method conforming the positions of these density peaks was used to normalize the hybridization data. Standard normalization methods for microarrays are unsuited for CGH data since the distribution of intensities between different hybridizations cannot be assumed to be thesame. Conforming the positions of density peaks is based on an assumptionthat all hybridizations exhibit high densities of both positive andnegative hybridization signals but does not alter the distribution patternof intensities. By positive and negative hybridization signals, we here mean signals representing present and absent/divergent genes, that is, high and low intensity signals, respectively. Visualization and clustering of data were conducted using MEV [47].

The distribution of logarithmic signal intensities was also used to set a threshold between the intensity peaks (lowest point of density) separately for each hybridization. This threshold was used to classify the probes and the corresponding genes as present, absent, or diverged in each strain. Intensity values of the threshold value ±0.3 were classified as diverged. The number of probes classified as "diverged" varied from 0 to 2 in all hybridizations, and these probes were considered as absent in further data analysis.

The data discussed in this paper are compliant with the MIAME guidelines and were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE67565 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67565).

2.5. Phylogenetic Analysis of Type VI Secretion System Sequences. Phylogenetic analysis similar to analysis described by Schwarz et al. [48] was performed on VipA sequences stored in the NCBI database and annotated to TIGR category COG3516 ($n = 195$). This TIGR sequence pool was supplemented by sequences annotated as VipA in *Y. pseudotuberculosis* IP32953 ($n = 4$), *B. thailandensis* ($n = 5$), *P. aeruginosa* ($n = 1$), *Y. enterocolitica* Y11 ($n = 1$), and *Y. enterocolitica* 8081 ($n = 1$). In total, 206 VipA sequences were aligned using MUSCLE [49] and the resulting alignment was visualized with BioNJ [50].

2.6. Orthologous Genes. Reciprocal Blast searches were performed to identify the bidirectional best hits between the genomes used in microarray design. Any bidirectional best hit identified was assumed to represent an orthologous gene pair [51]. Information on orthologous gene pairs was used as an aid in the interpretation of the microarray data.

3. Results

CGH analysis of 60 *Y. enterocolitica* and 38 *Y. pseudotuberculosis* strains was conducted with a DNA microarray based on three *Y. enterocolitica* and four *Y. pseudotuberculosis* genomes to shed light on genomic differences between enteropathogenic *Yersinia*. *Y. enterocolitica* strains and *Y. pseudotuberculosis* strains grouped into two distinct clusters (Figure 1). *Y. enterocolitica* strains belonging to four different biotypes formed distinct subclusters within the *Y. enterocolitica* group. Strains belonging to *Y. enterocolitica* biotype 2 or 3 ($n = 10$) clustered together (Figure 1). The distance between strains, based on Pearson's correlation on a scale from 0 to 2 (0 indicating identical samples), was 0.25 between *Y. enterocolitica* biotypes 2–4 and biotypes 1A and 1B. Within the *Y. pseudotuberculosis* group, the distance was 0.15. The distance between *Y. enterocolitica* and *Y. pseudotuberculosis* group was 1.36.

All hybridized strains produced positive signals on 459 probes, which is the equivalent of 320–360 genes depending on reference genome used. This means that around 8% of the genome is fully conserved across the two species. In the seven genomes used in array design, the core genome based on bidirectional best hits contained 2772 sequences, implying that 68–76% of genes in each sequenced genome have orthologous equivalents in the rest.

Comparing the 320 shared probes in hybridization results and 2772 in orthologous gene pairs, it becomes clear just how sensitive microarray hybridization is as a research method. Out of the 3547 probes (gene clusters) deemed present in all *Y. enterocolitica* strains, 1130 did not show a positive hybridization signal in any *Y. pseudotuberculosis* strain and were thus considered specific for *Y. enterocolitica* (Table S3). When these 1130 gene clusters were further pruned using the information on orthologous gene pairs, only 448 gene clusters remained truly specific for *Y. enterocolitica*. Similarly, in the *Y. pseudotuberculosis* group, 906 gene clusters were deemed conserved and specific (Table S3). *Y. enterocolitica* bioserotype 4/O:3 strains ($n = 42$) shared 51 gene clusters that were only extant in strains of this bioserotype. This represents around 1% of genes in the sequenced *Y. enterocolitica* 4/O:3 genome Y11.

Y. pseudotuberculosis strains shared three large operons coding type VI secretion systems that were missing from *Y. enterocolitica* strains. *Y. pseudotuberculosis* strains also shared a variety of gene clusters that based on their annotation are likely to be involved in the use and/or uptake of various substrates, including phenolic compounds, rhamnose, xylose, myoinositol, opines/polyamines, and aliphatic sulfonates (Table 1). *Y. enterocolitica* strains share six ATP-binding cassette (ABC) transporters and seven phosphotransferase systems (PTS) that are all absent from *Y. pseudotuberculosis* strains (Table 1). In addition, the *Y. enterocolitica* strains, excluding the highly virulent 1B strains, carry an

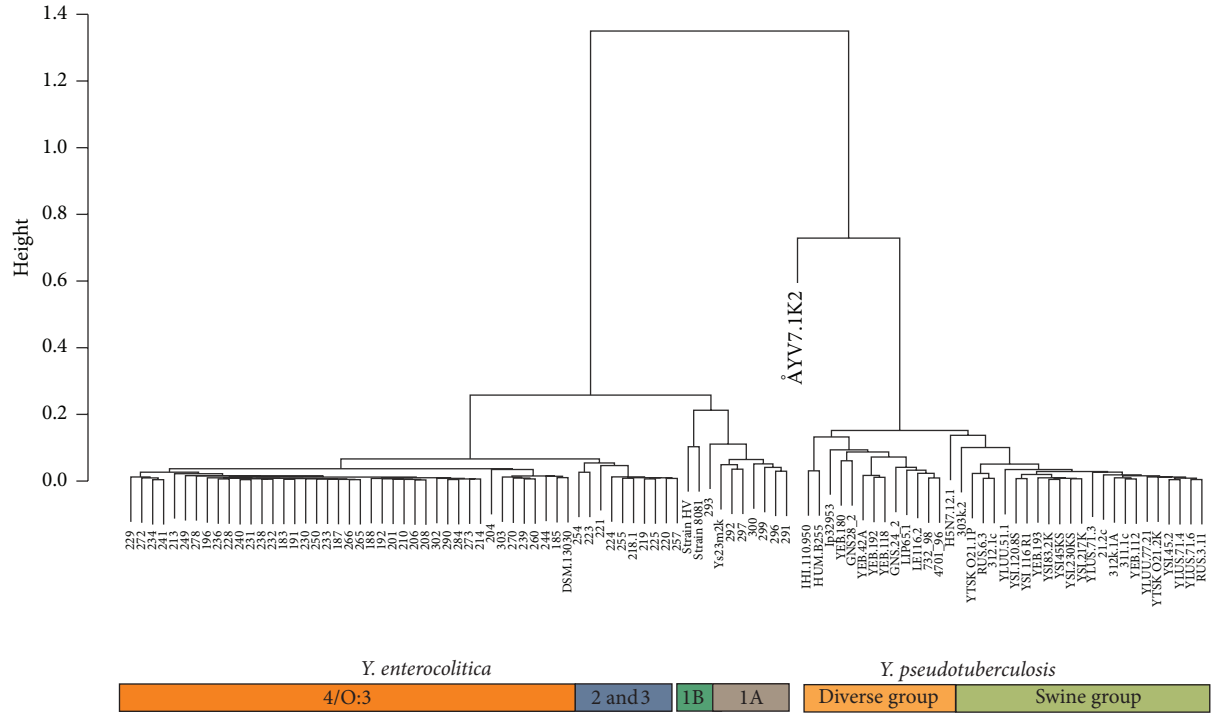

FIGURE 1: *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* strains group into two distinct clusters and *Y. enterocolitica* strains belonging to four different biotypes form distinct subclusters within the *Y. enterocolitica* group. The majority of *Y. pseudotuberculosis* strains obtained from swine samples cluster separately ("swine group") from strains obtained from human and wildlife samples ("diverse group"). The diverse group of *Y. pseudotuberculosis* also includes some strains isolated from English swine. Hierarchical clustering was constructed using R [40].

operon involved in the utilization of N-acetylgalactosamine. *Y. pseudotuberculosis* strains share 18 ABC transporters and 2 PTS transporters that are absent from *Y. enterocolitica* strains.

Phylogenetic analysis of the different T6SSs shows that three distinct types of T6SS are present in *Y. pseudotuberculosis* (Figure 2). One type present in two copies in *Y. pseudotuberculosis* (CAH19881.1, CAH21904.1 in *Y. pseudotuberculosis* IP32953) is present in one copy in *Y. enterocolitica* genomes (CAL12724.1 in strain 8081) and bears strong similarity to several T6SSs found in other bacteria. These include H1-T6SS found in *Pseudomonas aeruginosa* and four T6SSs found in *Burkholderia thailandensis*. Copies of the second type of T6SS (CAH20722.1 and CAH22490.1 in *Y. pseudotuberculosis* IP32953) clustered together with uncharacterized T6SSs found in *Y. pestis* (Figure 2). The function of T6SSs belonging to this type is unknown. The third distinct type of T6SS identified in *Y. pseudotuberculosis* genomes (CAH22876.1 in IP32953) shared strong similarity with the T6SSs of *Vibrio cholerae* and *B. thailandensis*, which are both considered to have cytotoxic effects against unicellular organisms and macrophages.

Relatively few gene cluster differences were observed between the hybridization results of different *Y. enterocolitica* strains (Figure 3). These included an operon coding for type III secretion system shared by low-pathogenic *Y. enterocolitica*, genes involved in drug resistance, and the operon coding from O:3 antigen. Many of the other differences are annotated as putative phages or flagellar components.

The majority of *Y. pseudotuberculosis* strains obtained from swine samples ("swine group" in Figure 1) in Finland, Sweden, Estonia, Russia, England, and Belgium ($n = 23$) clustered separately from human and wildlife samples ("diverse group" in Figure 1). Five of the 11 *Y. pseudotuberculosis* strains isolated from English pigs clustered together with the human and wildlife samples.

4. Discussion

Y. enterocolitica and *Y. pseudotuberculosis* strains grouped into two distinct clusters, and *Y. enterocolitica* strains belonging to four different biotypes formed distinct subclusters within the *Y. enterocolitica* group. On the gene level, the most interesting differences between *Y. enterocolitica* and *Y. pseudotuberculosis* strains included genes involved in T6SSs, the catabolism of phenolic compounds, and the transport of many carbohydrates (rhamnose, fructose, ribose, myoinositol, and xylose) and other compounds (aliphatic sulfonates, opines) (Table 1).

T6SS forms a needle-like injectisome between the bacterial cell and the target cell [52]. First described under ten years ago, T6SS is now one of the most common large specialized secretion systems found in over 120 bacteria [48, 53]. T6SSs

Table 1: Main differences in gene clusters between enteropathogenic *Yersinia enterocolitica* (YE) and *Yersinia pseudotuberculosis* (YP) strains.

Present in	Absent from	Locus	Gene names	Role	Description and comments
YE	YP	CBY25444.1-CBY25441.1	*aapJQMP*	Transportation	ABC transporter (L-amino acids).
YE	YP	CBY25938.1-CBY25944.1	*sorEMABF*	Transportation	Phosphotransferase system (sorbose) [21].
YE	YP, YE str. *8081*	CBY25947.1-CBY25953.1	*urtEDCBA*	Transportation	ABC transporter (urea). This copy of operon is absent from strain 8081.
YE	YP	CBY26058.1-CBY26056.1	*aglBA*	Transportation	Phosphotransferase system (alpha-glycosides).
YE	YP	CBY26159.1-CBY26152.1	—	Transportation	ABC transporter (metallic ion).
YE	YP	CBY26547.1-CBY26568.1	*pduVUTONMLKJBA*	Propanediol utilization	1,2-Propanediol utilization [21].
YE	YP	CBY26570.1-CBY26589.1	*cbiGKNQO*	Propanediol utilization	Cobalamin synthesis [21].
YE	YP	CBY26648.1-CBY26640.1	*citXFEDCAB*	Metabolism	Citrate lyase, ability to ferment citrate in anaerobic conditions.
YE	YP	CBY26805.1-CBY26815.1	*rutGEFDCGR*	Nitrogen metabolism	Pyrimidine utilization. Use of pyrimidines as a source of nitrogen in *E. coli*. Genes *rutA* and *rutG* are interrupted in *Y. enterocolitica*.
YE	YP	CBY28018.1-CBY28009.1	*scsBCD*	Resistance	Suppressor for copper sensitivity operon 2. Similar operon described in *E. coli*.
YE	YP	CBY28023.1-CBY28018.1	—	Transportation	Phosphotransferase system (lactose/cellobiose).
YE	YP	CBY28059.1-CBY28057.1	*ascGFB*	Transportation	Phosphotransferase system (β-glycosides).
YE	YP	CBY28068.1-CBY28065.1	*yrbFE*	Transportation	ABC transporter (YrbF/E).
YE	YP	CBY28213.1-CBY28205.1	*gutQRMDBEA*	Transportation	Phosphotransferase system (glucitol/sorbitol).
YE	YP	CBY28759.1-CBY28735.1	*gldA, dhaKR, scrRBAYK*	Metabolism, transportation	Glycerol metabolism operon, phosphotransferase system (sucrose).
YE	YP	CBY29405.1-CBY29415.1	*bcsCBAFEG*	Gut colonization	Cellulose biosynthesis [21].
YE	YP	CBY29454.1-CBY29443.1	*manA, bglA, gmuD*	Transportation	Phosphotransferase system (lactose/cellobiose), maltoporin, and β-glucosidase
YP	YE	CAH19316.1-CAH19320.1	—	Transportation	ABC transporter (molybdate-malate).
YP	YE	CAH19585.1-CAH19591.1	—	Resistance	Methyltetrahydrofolate reduction, conserved with ter operon
YP	YE	CAH19592.1-CAH19597.1	*terZABCDE*	Resistance	Tellurite/tellurium resistance. Similar to the operon in *Y. pestis* plasmid.
YP	YE	CAH19782.1-CAH19785.1	*frwDBC, pstA*	Transportation	Phosphotransferase system (fructose).
YP	YE	CAH19879.1-CAH19896.1	*impACG, hcp, vasG, icmF*	Type VI secretion	YPTB T6SS-1, interbacterial interaction.
YP	YE	CAH20037.1-CAH20044.1	*sgbK, araD*	Transportation	ABC transporter (L-xylose), epimerase.
YP	YE	CAH20283.1-CAH20290.1	—	Unknown	CDP-diacylglycerol synthesis operon. A similar gene cluster of unknown function has been described in *E. coli*.
YP	YE	CAH20313.1-CAH20316.1	—	Transportation	ABC transporter (myoinositol), dehydrogenase.

Table 1: Continued.

Present in	Absent from	Locus	Gene names	Role	Description and comments
YP	YE	CAH20560.1-CAH20567.1	*rpiA*	Transportation	ABC transporter (sugar), dehydrogenase.
YP	YE	CAH20608.1-CAH20613.1	—	Transportation	ABC transporter (iron).
YP	YE	CAH20708.1-CAH20725.1	—	Type VI secretion	Conserved area before type VI secretion system.
YP	YE	CAH20725.1-CAH20742.1	*impKL, hcp, vasGD*	Type VI secretion	YPTB T6SS-2.
YP	YE	CAH20812.1-CAH20822.1	*lidD*	Transportation	Symport.
YP	YE	CAH20875.1-CAH20884.1	*hpaRGEDFHIXBC*	Use of aromatic substances	Hpa operon.
YP	YE	CAH20923.1-CAH20928.1	—	Transportation	ABC transporter (sugar).
YP	YE	CAH21145.1-CAH21154.1	—	Transportation	ABC transporter (sorbitol).
YP	YE	CAH21162.1-CAH21172.1	—	Transportation	MFS transporter (aromatic acids).
YP	YE	CAH21251.1-CAH21255.1	*potDCBA*	Transportation	ABC transporter (polyamines).
YP	YE	CAH21445.1-CAH21448.1	*tauB*	Transportation	Transporter (taurine/sulfonate).
YP	YE	CAH21739.1-CAH21760.1	*manB, mtlK*	Transportation	MFS transporter (sugar), ABC transporter (sugar), and CRISPR repeats.
YP	YE	CAH21766.1-CAH21777.1	*gutB*	Transportation	Carnitine transporter, tartrate dehydrogenase, and ABC transporter (sorbitol).
YP	YE	CAH22045.1-CAH22050.1	*goaG*	Transportation	ABC transporter (opines/polyamines).
YP	YE	CAH22292.1-CAH22299.1	*gspLKJHI*	Type II secretion	General secretion pathway.
YP	YE	CAH22307.1-CAH22312.1	—	Growth on chondroitin sulfate	Secreted chondroitin ABC lyase.
YP	YE	CAH22317.1-CAH22328.1	*kduD2*	Transportation	Phosphotransferase system (N-acetylgalactosamine), chondro-6-sulfatase.
YP	YE	CAH22333.1-CAH22353.1	*lamb, bgaB*	Transportation	ABC transporter (maltodextrin/maltose/ribose).
YP	YE	CAH22467.1-CAH22469.1	*mglA*	Transportation	ABC transporter (sugar).
YP	YE	CAH22662.1-CAH22687.1	*yapF*	Transportation	Na+/H+-antiport, ABC transporter (sugar).
YP	YE	CAH23038.1-CAH23046.1	—	Transportation	ABC transporter (ribose), two-component system.
YE 4/O:3	YE BT 1–3, YP	CBY26503.1-CBY26517.1		Serotype O:3 antigen	dDTP-L-rhamnose biosynthesis [22, 23].
YE 4/O:3	YE BT 1–3, YP	CBY26512.1-CBY26517.1		Serotype O:3 antigen	Conserved area posterior to the O:3 antigen. Hypothetical proteins, transposon.
YE BT 2–4	YE BT 1A, 1B; YP	CBY25728.1-CBY25740.1	*aatBCAP, araC*	Resistance	Multidrug efflux system. Cluster includes 6 genes and 7 hypothetical insertion sequences.
YE BT 2–4, 1	YE BT 1A, 1B; YP	CBY29000.1-CBY29007.1	*sseDBCEBF*	Virulence, type III secretion	Type III secreting effectors and chaperones. *Salmonella* type III secretion Sse operon is involved in interaction with macrophages.

Table 1: Continued.

Present in	Absent from	Locus	Gene names	Role	Description and comments
YE BT 2–4, 1A	YE BT 1B	CBY28981.1-CBY29013.1	*ysp*	Virulence, type III secretion	Not fully conserved in biotype 1A strains [24].
YE BT 2–4, 1A	YE BT 1B	CBY26978.1-CBY26985.1	*agaRZVWEFSY*	N-Acetylgalactosamine utilization	Use of intestinal mucin as a carbon source [24].

YE = *Y. enterocolitica*, YP = *Y. pseudotuberculosis*, BT = biotype(s).

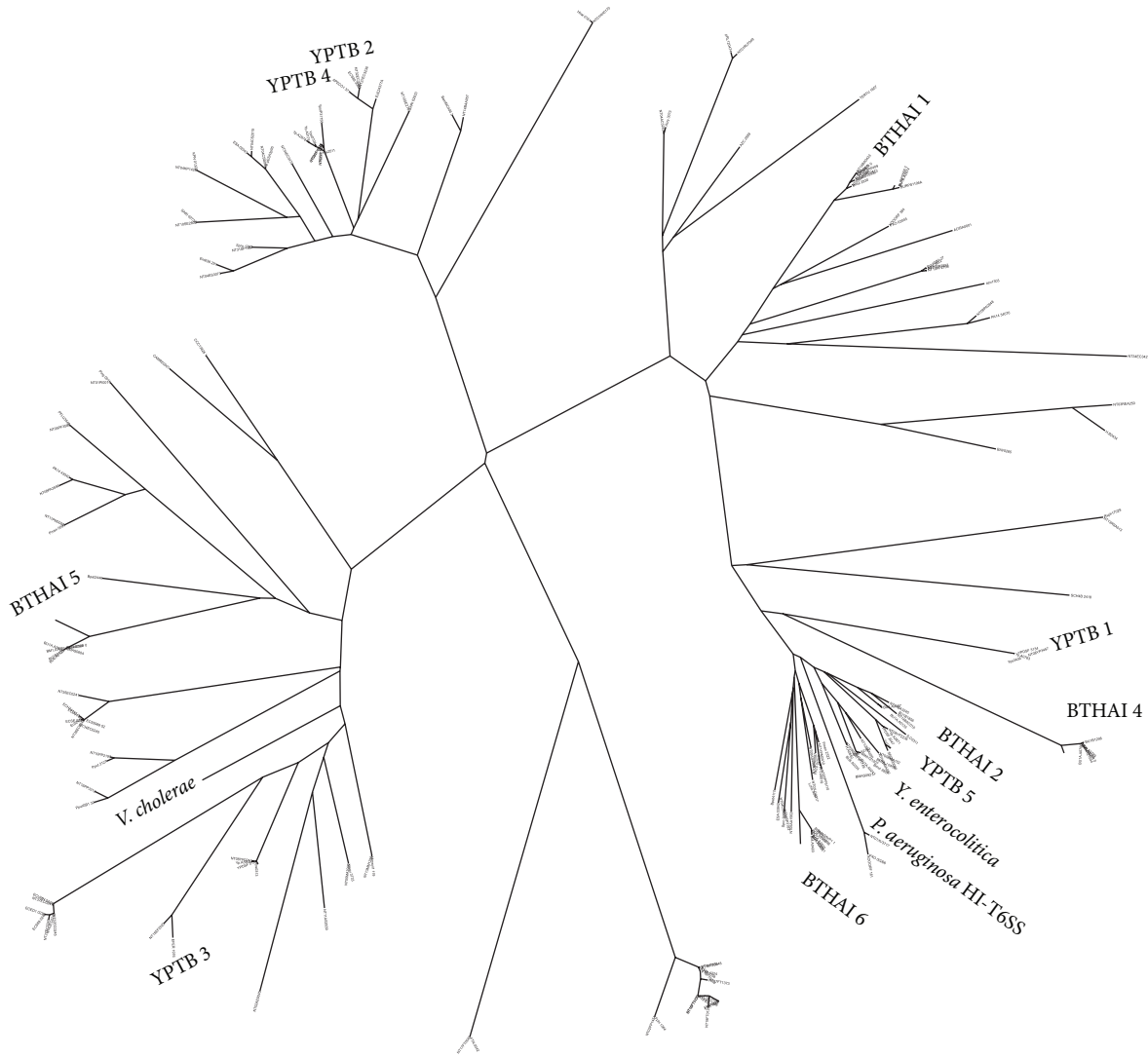

Figure 2: Phylogenetic relationships of type VI secretion systems (T6SSs) in *Yersinia pseudotuberculosis* and T6SSs of other species were compared to evaluate the different types of T6SS. VipA sequences were used to represent T6SS and the alignment of 206 VipA proteins is shown here as an unrooted phylogenetic tree visualized by BioNJ. Type VI secretion systems of *Y. pseudotuberculosis* named YPTB 1–5 belong to three distinct branches of T6SSs. T6SSs of *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Burkholderia thailandensis* (BTHAI 1–6) are marked on the branches of the phylogenetic tree and the one VipA/T6SS present in *Y. enterocolitica* is also shown.

were first considered as virulence factors, but their abundance in nonpathogenic bacteria and further studies have suggested that most of these systems play a role in interbacterial interaction and defense against competitive bacteria and unicellular organisms [54]. The mechanism requires 15 conserved genes and direct contact with other cells and is thus thought to be especially useful in the stationary growth phase [52, 55, 56]. It is notable that diverse collections of T6SSs have been reported in many environmental bacteria with facultative pathogenic potential, such as *Pseudomonas aeruginosa*, *Burkholderia*

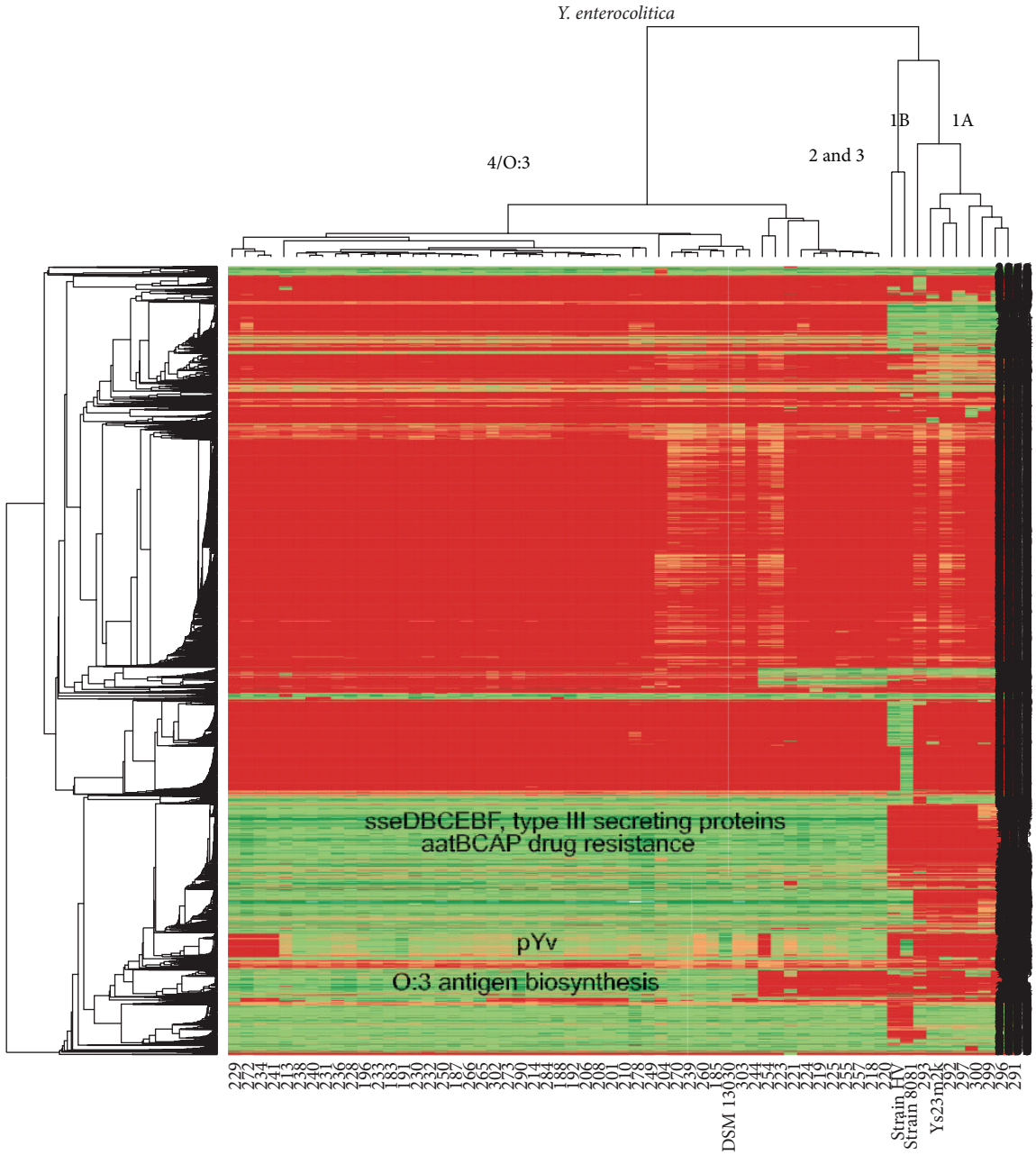

Figure 3: A heatmap presentation of *Yersinia enterocolitica* hybridization results produced with R. Biotypes cluster separately and major gene clusters differing between different biotypes are shown. A green color signifies that the gene is present in the given strain.

mallei, and *Burkholderia pseudomallei*, as well as in bacteria with multiple hosts and the ability to survive in diverse environmental conditions (*Y. pestis*, *V. cholerae*) [53, 57–60]. *Y. pseudotuberculosis* is also considered a facultative pathogen with multiple host species and able to persist in the environment. The genome of *Y. pseudotuberculosis* has four conserved systems and one smaller, perhaps partial system [55]. Only one of these systems is shared with *Y. enterocolitica*. The loci coding VipA protein is used to indicate the location of each T6SS. Two of the *Y. pseudotuberculosis* T6SSs (CAH19881.1, CAH21904.1 in IP32953) and the solitary T6SS in *Y. enterocolitica* (CAL12724.1 in strain 8081) group in phylogenetic analysis together with *B. thailandensis* T6SS (BTHAI-1) and H1-T6SS of *P. aeruginosa*. The latter two have been reported to target other bacteria and give some competitive advantage to the bacterium itself [48, 56]. Having this mechanism could enhance the growth of *Y. pseudotuberculosis* when other bacteria are present on a shared growth surface. Another T6SS (CAH22876.1 in IP32953) is similar to the T6SSs in *V. cholerae* and *B. thailandensis*, which are described as being cytotoxic against single-celled organisms and macrophages [48, 53, 61]. T6SSs like this are beneficial against protists living in the soil and water environment but are also possible pathogenicity factors. To better understand

the function of each T6SS of *Y. pseudotuberculosis*, *in vivo* studies are required. Epidemiologically, T6SSs could probably help *Y. pseudotuberculosis* to survive and multiply in such ecological niches in the environment from which it could easily end up as a contaminant of the food chain. The lack of T6SSs in *Y. enterocolitica* implies that the organism in its current ecological niche has no need for them. The lack of T6SSs might actually be beneficial for the organism, as a T6SS with cytotoxic effects against the macrophages of the mammal host might encumber the invasion and survival of *Y. enterocolitica* cells.

Y. pseudotuberculosis strains also carry a variety of gene clusters involved in the uptake and/or utilization of various substrates (Table 1). The Hpa operon (CAH20875.1–CAH20884.1 in IP32953), also known as the 4-hydroxyphenylacetate degrading operon, is involved in the catabolism of phenolic and aromatic compounds [62, 63]. Based on database queries, Hpa sequences in *Y. pseudotuberculosis* are homologous to those in *E. coli* and *Salmonella*. Phenols are products of plant secondary metabolism and often have bactericidal effects. Phenols are widely present in soil and the water environment, but their abundance in the intestines of animals has also been suggested [62, 63]. Interestingly, the hpa genes are expressed in *Salmonella enterica* serovar Typhimurium cells during the infection in swine, and it has been suggested that the operon is somehow beneficial for enteropathogenic bacteria [64]. Evidently, the lack of an Hpa operon does not seem to hinder the prevalence of *Y. enterocolitica* in swine.

Both *Y. enterocolitica* and *Y. pseudotuberculosis* have many transport and uptake systems that are not present in other species. *Y. enterocolitica* strains share six ABC transporters and seven PTS transporters that are all absent from *Y. pseudotuberculosis* strains. Putative substrates for these systems include iron and metallic ions, glycosides, and lactose/cellobiose. Interestingly, one putative urea ABC transporter was noted as present in all other *Y. enterocolitica* strains, but absent from reference strain 8081. This highlights the benefits of having multiple reference strains.

Y. enterocolitica strains shared notably fewer specific genes ($n = 448$) between them than *Y. pseudotuberculosis* strains ($n = 906$). This is likely to reflect the greater heterogeneity within *Y. enterocolitica* biotypes and the ecological adaptation of biotypes 2–5 by gene loss and genome decay [4]. Many of these specific genes were involved in transportation. *Y. pseudotuberculosis* strains shared 18 ABC transporters and 2 PTS transporters absent from *Y. enterocolitica*. Putative substrates for these systems include rhamnose, fructose, xylose, myoinositol, iron, aromatic acids, polyamines, sorbitol, sulfonates, and 7 systems for unspecified sugars. The *Y. pseudotuberculosis* genome appears to be equipped with many extra tools for moving substances in and out of its cell compared to the *Y. enterocolitica* genome, which appears more streamlined and likely to have adapted to another ecological niche, such as swine tonsils and gut, where variety in substrate transportation is not required. A recent hypothesis on the evolution of enteropathogenic *Yersinia* assumes that *Yersinia* species have evolved to become more ecologically specific and metabolically more limited to their reservoirs by genome decay and gene loss [4]. An evolutionary path such as this appears plausible, as *Y. enterocolitica* seems better adapted to living in a mammal host and to have lost many genes involved in survival in the environment.

The theory of genome decay and gene loss, however, does not explain the differences between *Y. enterocolitica* biotypes. Lipase activity, hydrolysis of B-glycosides such as salicin and esculin, use of xylose, and indole production are some of the biochemical tests belonging to *Y. enterocolitica* biotyping schema [2]. However, relatively few clusters of genes differentiate pathogenic and nonpathogenic *Y. enterocolitica* strains from each other. Recent results have identified the changes in gene expression patterns for pathogenicity factors explaining the swine specificity of *Y. enterocolitica* 4/O:3 [22]. It seems likely that the adaptation and differences in pathogenicity of *Y. enterocolitica* biotypes are due to point mutations and changes in gene expression rather than gene loss.

Y. pseudotuberculosis strains obtained from swine samples mostly clustered separately from human and wildlife samples (Figure 1). Notably, five of the 11 *Y. pseudotuberculosis* strains isolated from English swine clustered together with the human and wildlife samples. Niskanen et al. [39] have previously reported on the homogeneity of *Y. pseudotuberculosis* strains isolated from swine samples based on pulsed-field gel electrophoresis analysis. Our results further confirm this finding. Martínez et al. [25–27] noted that the prevalence and diversity of *Y. pseudotuberculosis* strains appears to be higher in English swine than in swine of other European countries. This finding is also supported by the present results, as 5 of the 11 English *Y. pseudotuberculosis* strains showed a marked genetic distance to other swine strains. In these results, no defining gene cluster setting the swine group and diverse group apart could be identified. In this type of study, the results are dependent on the reference strains used. It is important to note that because of the limitations of the method, many genes present in the studied strains might be absent from the reference genomes used and thus from the designed microarray and the further results. It would be interesting to have a wholly sequenced genome from the "swine group" of *Y. pseudotuberculosis* for further research on the differences between these two groups.

The high prevalence of *Y. pseudotuberculosis* in the English pork chain is probably explained by the more available access to outdoors of English swine compared to their continental counterparts. Swine and pork products are not considered to be a notable source of sporadic *Y. pseudotuberculosis* cases, and animals having greater contact with the environment are more likely to have strains of soil and wildlife origins passing through their intestines. This would also explain why some *Y. pseudotuberculosis* not belonging to the "swine group" of *Y. pseudotuberculosis* have been isolated from English swine.

5. Conclusions

The hybridization results revealed that *Y. pseudotuberculosis* strains carry many operons linked with the use of carbohydrates and other substrates that are absent from *Y. enterocolitica*. Phenolic compounds, polyamines, myoinositol, and

aliphatic sulfonates are all substrates that are not commonly present in living animal tissue but are more abundant in soil and the environment. *Y. pseudotuberculosis* also harbors an array of different type VI secretion systems, in contrast to just one found in the *Y. enterocolitica* genome. Type VI secretion systems target single-celled organisms and other bacteria but are also possible pathogenicity factors. These defense and interaction systems could help *Y. pseudotuberculosis* to survive and multiply in such ecological niches in the environment from which it could easily end up as a contaminant of the food chain.

The *Y. pseudotuberculosis* genome holds many tools, such as type VI secretion systems and transporters for various substrates, which are likely to be beneficial for survival in varied growth environments and multiple host species. By comparison, the genome of *Y. enterocolitica* appears more streamlined and likely to have adapted to a different ecological niche where these survival systems are not needed or beneficial. For *Y. enterocolitica* bioserotype 4/O:3, this niche is with certainty swine.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Comparison of Antibiotic Resistance and Virulence Factors among *Escherichia coli* Isolated from Conventional and Free-Range Poultry

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Microbiological contamination in commercial poultry production has caused concerns for human health because of both the presence of pathogenic microorganisms and the increase in antimicrobial resistance in bacterial strains that can cause treatment failure of human infections. The aim of our study was to analyze the profile of antimicrobial resistance and virulence factors of *E. coli* isolates from chicken carcasses obtained from different farming systems (conventional and free-range poultry). A total of 156 *E. coli* strains were isolated and characterized for genes encoding virulence factors described in extraintestinal pathogenic *E. coli* (ExPEC). Antimicrobial susceptibility testing was performed for 15 antimicrobials, and strains were confirmed as extended spectrum of β-lactamases- (ESBLs-) producing *E. coli* by phenotypic and genotypic tests. The results indicated that strains from free-range poultry have fewer virulence factors than strains from conventional poultry. Strains from conventionally raised chickens had a higher frequency of antimicrobial resistance for all antibiotics tested and also exhibited genes encoding ESBL and AmpC, unlike free-range poultry isolates, which did not. Group 2 CTX-M and CIT were the most prevalent ESBL and AmpC genes, respectively. The farming systems of poultries can be related with the frequency of virulence factors and resistance to antimicrobials in bacteria.

1. Introduction

Resistance to antimicrobial agents has become a major concern both for human health and in veterinary medicine. Antimicrobial agents are being used in many countries in veterinary practice for therapy and prophylaxis of infectious diseases and for growth promotion in food animals. However, the indiscriminate use of antimicrobials can result in bacterial selection pressure of the intestinal microbiota of animals [1–3]. Because multiresistant bacteria are frequently found in poultry meat [4–6], chicken products are suspected to be a source of foodborne pathogen and/or antimicrobial resistance bacteria for humans [1–3, 7, 8].

Escherichia coli have an important role within resistant bacteria populations, being widely used as a bioindicator of antimicrobial resistance and being pathogenic to humans and animals. Extraintestinal pathogenic *Escherichia coli* (ExPEC) can cause many human infections, such as septicemia, meningitis, and urinary tract infections, and can also cause disease in birds, being responsible for significant economic

losses in poultry industry [1, 9]. ExPECs are characterized by the possession of many virulence factors including adhesins, toxins, iron acquisition systems, and serum resistance factors and, in phylogenetic classification, belong mainly to group B2 and occasionally to group D, whereas commensal *E. coli* belong to groups B1 and A [10, 11].

β-lactamase production is the most common mechanism of resistance for β-lactam in Gram-negative bacteria and is increasing in occurrence in humans, becoming a major public health problem [9]. However, β-lactamases of community and environmental origin have been discovered, for example, in food animals. Poultry are recognized as important carriers of β-lactamase-producing *E. coli*, and extended-spectrum β-lactamase (ESBL)/AmpC-producing bacteria in birds have been reported in many countries [12–14].

ESBL production confers resistance to 3rd- and 4th-generation cephalosporins but not to cephamycins (cefoxitin) and carbapenems and is inactivated by clavulanic acid. The AmpC enzymes confer resistance to 3rd-generation cephalosporins and cephamycins but are inhibited by β-lactamase inhibitors. Plasmid-mediated β-lactamases can carry multiple resistance genes non-β-lactaman, and their indiscriminate use can lead to coselection and/or coresistance in bacteria populations [9, 13, 15].

Many studies reported that there is a genetic similarity among avian and human ExPEC, leading to the hypothesis that meat animals play a role as reservoirs for drug-resistant bacteria and pathogenic bacteria [1, 16]. Little is known regarding the microbiological quality of chicken meat from different systems of poultry farming and their potential antimicrobial resistance and/or pathogenic behavior upon consumption. The aim of this study was to analyze the profile of virulence factors and antimicrobial resistance, including searching for ESBL/AmpC groups genes, in strains of *E. coli* isolated from conventional and free-range poultry carcass.

2. Material and Methods

2.1. Bacterial Isolates. A total of 156 *E. coli* strains were isolated from commercial refrigerated chicken carcass, intended only for local consumption, sold in the city of Londrina (north region in Paraná, Brazil). Of these, 35 *E. coli* strains were isolated from 15 free-range poultry (commonly created by family agriculture) and 121 *E. coli* strains from 26 conventionally raised poultry (sold in markets in the region, obtained from granges) [17]. Each chicken carcass was placed into the sterile packaging with 100 mL of Brain Heart Infusion (Himedia Laboratories Pvt. Ltd., Mumbai, India). After homogenization, 0.1 mL was smeared onto MacConkey agar (Neogen Corporation Lansing, Michigan) and crystal violet red neutron bile agar (Neogen Corporation Lansing, Michigan) by pour plate. Both were incubated at 37°C for 18–24 h. Colonies suspected to be *E. coli* were confirmed by biochemical tests such as EPM, MILi [18, 19], and Simons citrate agar (Merck, KGaA, Darmstadt, Germany). One-to-eight strains were collected from each chicken carcass. Only strains that showed different genotypic characteristics of virulence factors and phenotypic resistance were selected.

2.2. Phylogenetic Classification. *E. coli* strains were assigned to phylogenetic groups (A, B1, B2, or D), according to the method of Clermont and collaborates [10]. This method is based on analysis of presence of the *chu*A and *yja*A genes and the DNA fragment (TSPE4.C2), as determined by Polymerase Chain Reaction (PCR). This PCR reaction contained 1.25 U Taq DNA polymerase (Life technologies, Rockville, MD) in 1x PCR buffer (Life technologies, Rockville, MD), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 1 μM of each primer. The conditions of PCR consisted of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension step at 72°C for 7 min. PCR amplicons were visualized on 2.0% agarose gels stained with GelRed (Biotium, Hayward, CA, USA). After gel electrophoresis, the images were captured using Image Capture Systems (LPixImageHE).

2.3. Virulence Factor Genes. Several virulence factors normally studied in ExPEC strains were surveyed. The selected genes were as follows: *iut*A (aerobactin siderophore receptor gene), *hly*F (putative avian hemolysin), *iss* (episomal increased serum survival gene), *iro*N (salmochelin siderophore receptor gene), and *omp*T (episomal outer membrane protease gene) [11]. This PCR contained 1.25 U Taq DNA polymerase (Life technologies, Rockville, MD) in 1x PCR buffer (Life technologies, Rockville, MD), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, and 1 μM of each primer. The conditions of PCR consisted of 94°C for 2 min, followed by 25 cycles of 94°C for 30 s, 63°C for 30 s, and 68°C for 3 min, with a final extension step at 72°C for 10 min [11]. PCR amplicons were visualized on 2.0% agarose gels stained with GelRed (Biotium, Hayward, CA, USA). After gel electrophoresis, the images were captured using Image Capture Systems (LPixImageHE).

2.4. Antimicrobial Susceptibility Testing. Antimicrobial susceptibility was performed using the standard disk diffusion method recommended by the Clinical and Laboratory Standards Institute [20, 21]. Antimicrobials used included 5 μg of ciprofloxacin, 10 μg of each of ampicillin, gentamicin, norfloxacin, and enrofloxacin, 30 μg of each of cefazolin, cefotaxime, cefoxitin, ceftazidime, tetracycline, nalidixic acid, and chloramphenicol, 300 μg of nitrofurantoin, 1.25/23.75 μg of trimethoprim-sulfamethoxazole, and 20/10 μg of amoxicillin-clavulanic acid (Oxoid Ltd., Basingstoke, Hants, UK). Strains resistant to third-generation cephalosporins were confirmed for ESBL production by double-disk diffusion testing between amoxicillin/clavulanate and cefotaxime or ceftazidime [22], or by using a combination disc test including cefotaxime, cefotaxime + clavulanic acid (Becton Dickinson, Sparks, MD), ceftazidime, and ceftazidime + clavulanic acid (Becton Dickinson, Sparks, MD), according to the CLSI recommendations. The strains positive in the phenotypic tests to ESBL production were screened to ESBL genes. Strains that showed cefoxitin and/or to 3rd-generation cephalosporins intermediate or resistance were tested by molecular screening of AmpC type genes. The *E. coli* isolate ATCC 25922 was used as a quality control to antimicrobial susceptibility testing, and the results were interpreted as per CLSI criteria.

2.5. Characterization of β-Lactamase Genes of ESBL and AmpC Groups. ESBL-producing *E. coli* was characterized for ESBL genes encoding CTX-M (1, 2, 8, 9, and 25 groups), TEM, and SHV type by PCR [23–25]. All isolates suspected by phenotypic tests for the production of AmpC were tested by a multiplex PCR described by Pérez-Pérez and Hanson [26]. Six family-specific plasmid mediated AmpC genes (MOX, FOX, EBC, ACC, DHA, and CIT) were evaluated. PCR amplicons were visualized on 2.0% agarose gels stained with GelRed (Biotium, Hayward, CA, USA). After gel electrophoresis, the images were captured using Image Capture Systems (LPixImageHE).

2.6. Statistical Analysis. Comparisons of frequencies among different groups were made by Fisher's exact test and Chi-square test. Findings were considered to be significant where $p < 0.05$. The test was performed with the statistical program R version 3.1.0.

3. Results

According to phylogenetic classification, the most prevalent group in strains from free-range poultry was the group A (54.3%), whereas the strains from conventionally raised poultry most frequently belonged to group B1 (37.2%), although no statistically significant differences were observed between them and groups B1, B2, and D (Table 1).

Regarding the search for virulence factors, we found significant difference for the majority of the genes studied between strains from free-range and conventional poultry, with the exception of the *iss* gene ($p > 0.05$) (Table 1). Few strains from free-range poultry were positive for virulence factors, with only 10 strains (28.6%) having at least one of the virulence factors studied. In contrast, 91 strains (75.2%) from conventionally raised poultry had at least one virulence factor.

According to the antimicrobial susceptibility test, strains from conventionally raised poultry showed a higher frequency of antimicrobial resistance than strains from free-range poultry for all antimicrobials tested (Figure 1). The frequency of antimicrobial resistance to strains from free-range poultry was low, except to tetracycline (60% of resistance), whereas the strains from conventional poultry showed a high frequency of resistance mainly to tetracycline, nalidixic acid, and ampicillin (Figure 1).

ESBL/AmpC genes appeared only in strains isolated from conventional poultry (42.1% of 121 strains from conventional poultry). Forty strains were ESBL-producing *E. coli*. The most prevalent group within these ESBL was the group 2 CTX-M (62.5% of ESBL-producing strains). Eleven strains showed only the CIT group of AmpC genes (9.1% of 121 strains from conventional poultry). No strain had ESBL and AmpC genes together (Table 2).

All ESBL/AmpC-producing strains showed resistance to one or more non-β-lactam antimicrobials, with resistance to tetracycline (98%) the most prevalent (Table 2).

We observed that ESBL/AmpC-producing strains were present in all four phylogenetic groups (A, B1, D, and B2), although there were few B2 strains. The majority of these strains were positive for at least one virulence factor.

Table 1: Prevalence of phylogenetic group and virulence genes in strains of *E. coli* isolated by free-range and conventionally raised poultry carcass.

	Free-range ($n = 35$)	Conventional ($n = 121$)
	Number of isolates (%)	Number of isolates (%)
Phylogenetic group		
A	19 (54.3)*	35 (28.9)
B1	09 (25.7)	45 (37.2)
B2	00 (0)	5 (4.1)
D	07 (20)	36 (29.7)
Virulence genes		
hlyF	09 (25.7)	57 (47.1)*
iutA	06 (17.1)	66 (54.5)*
iss	07 (20)	43 (35.5)
*omp*T	08 (22.9)	64 (52.9)*
*iro*N	01 (2.9)	35 (28.9)*

*$p < 0.05$, Chi-square. Free-range versus conventionally raised poultry carcass *E. coli* isolates.

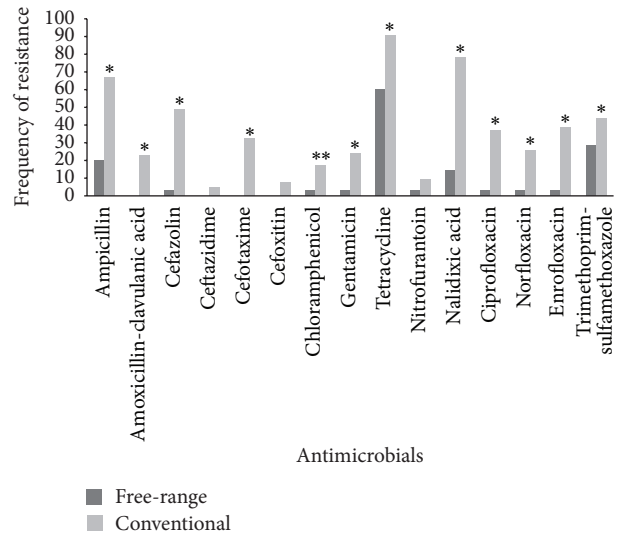

Figure 1: Frequency of antimicrobial resistance to *E. coli* strains isolated from free-range and conventional chicken carcass. *$p < 0.05$, Chi-square test. **$p < 0.05$, Fisher exact test.

4. Discussion

Many studies have demonstrated similarities between human and avian ExPEC, leading to the hypothesis that poultry products may serve as a source of ExPEC and are closely linked to human infections. Poultry meat exhibits the highest levels of *E. coli* contamination, and these are indicated as being more extensively antimicrobial-resistant than *E. coli* from other meats [27].

Table 2: Characteristics of β-lactamase genes and phenotypic antimicrobial resistance profile of strains of ESBL/AmpC-producing *E. coli*.

Isolate number	Phenotypic resistance profile	β-lactamase genes
1	Amp, amc, cfz, ctx, tet, nal	Group 1 CTX-M
2	Amp, kz, ctx, cn, tet, nal	Group 2 CTX-M
3	Amp, kz, ctx, cn, tet, nal, cip, nor, enr, sut	Group 2 CTX-M
4	Amp, kz, ctx, cn, tet, nal, cip, sut	Group 2 CTX-M
5	Amp, kz, ctx, cn, tet, nal, cip, enr	Group 2 CTX-M
6	Amp, kz, ctx, cn, tet, nal	Group 2 CTX-M
7	Amp, kz, ctx, clo, cn, nal, sut	Group 2 CTX-M
8	Amp, kz, ctx, tet, nal, cip, nor, enr, sut	Group 2 CTX-M
9	Amp, kz, ctx, tet, nal, cip, nor, enr, sut	Group 2 CTX-M
10	Amp, kz, ctx, clo, tet, nal	Group 2 CTX-M
11	Amp, kz, ctx, cn, tet, nal, cip, enr, sut	Group 2 CTX-M
12	Amp, kz, ctx, cn, tet, nal, enr	Group 2 CTX-M
13	Amp, kz, ctx, cn, tet, nal	Group 2 CTX-M
14	Amp, kz, ctx, cn, tet, nal, cip, sut	Group 2 CTX-M
15	Amp, amc, kz, cn, tet, nal, sut	Group 2 CTX-M
16	Amp, amc, kz, ctx, cn, tet, nal	Group 2 CTX-M
17	Amp, amc, kz, ctx, cn, tet, nal, cip, nor, enr, sut	Group 2 CTX-M
18	Amp, kz, ctx, cn, tet, nal	Group 2 CTX-M
19	Amp, kz, ctx, cn, tet, nal	Group 2 CTX-M
20	Amp, kz, ctx, clo, tet, nal, cip, nor, sut	Group 2 CTX-M
21	Amp, kz, ctx, cn, tet, nal, cip, enr	Group 2 CTX-M
22	Amp, amc, kz, ctx, tet, nit, nal	Group 2 CTX-M
23	Amp, amc, kz, ctx, cn, tet	Group 2 CTX-M
24	Amp, amc, kz, ctx, clo, tet, nit, nal, cip, nor, enr, sut	Group 8 CTX-M
25	Amp, kz, ctx, tet, enr	Group 8 CTX-M
26	Amp, kz, ctx, tet, nit	Group 8 CTX-M
27	Amp, amc, kz, ctx, clo, tet, nal, sut	Group 8 CTX-M
28	Amp, kz, ctx, tet	Group 8 CTX-M
29	Amp, kz, ctx, tet, nal, cip, nor, enr	Group 8 CTX-M
30	Amp, kz, ctx, tet	Group 8 CTX-M
31	Amp, kz, ctx, clo, tet, nal, cip, nor, enr	Group 8 CTX-M
32	Amp, kz, ctx, tet	Group 8 CTX-M
33	Amp, kz, ctx, caz, tet	Group 8 CTX-M
34	Amp, kz, ctx, tet, nit	Group 8 CTX-M
35	Amp, kz, ctx, tet, nal, cip, nor, enr	SHV
36	Amp, amc, kz, clo, cn, tet, nit, sut	CIT
37	Amp, amc, kz, cfo, tet, nal, sut	CIT
38	Amp, amc, kz, cfo, cn, tet, nal, sut	CIT
39	Amp, amc, kz, cfo, caz, tet, nal, sut	CIT
40	Amp, amc, kz, cfo, tet, nal, sut	CIT
41	Amp, amc, kz, cfo, caz, tet, nal, cip, nor, enr, sut	CIT
42	Amp, amc, kz, cfo, tet, nal, sut	CIT
43	Amp, amc, kz, cfo, clo, cn, tet, nal, cip, nor, enr, sut	CIT
44	Amp, amc, kz, cfo, ctx, tet, nal, sut	CIT
45	Amp, amc, kz, cfo, caz, tet, nal, cip, enr, sut	CIT
46	Amp, amc, kz, cfo, caz, tet, nit, nal, cip, enr	CIT
47	Amp, amc, kz, ctx, caz, clo, tet, nal, cip, nor, enr, sut	Group 1 CTX-M, Group 2 CTX-M
48	Amp, kz, ctx, tet, nal, cip, nor, enr, sut	Group 2 CTX-M, Group 8 CTX-M
49	Amp, amc, kz, ctx, tet, nal	Group 8 CTX-M, SHV

Table 2: Continued.

Isolate number	Phenotypic resistance profile	β-lactamase genes
50	Amp, amc, kz, tet, nal	Group 8 CTX-M, SHV
51	Amp, amc, kz, ctx, clo, tet, nal, cip, nor, enr, sut	Group 2 CTX-M, Group 8 CTX-M, SHV

Ampicillin (AMP); amoxicillin-clavulanic acid (AMC); cefazolin (KZ); ceftazidime (CAZ); cefotaxime (CTX); chloramphenicol (CLO); gentamicin (CN); tetracycline (TET); nitrofurantoin (NIT); nalidixic acid (NAL); ciprofloxacin (CIP); norfloxacin (NOR); enrofloxacin (ENR); trimethoprim-sulfamethoxazole (SUT); not found (NF).

Avian *E. coli* often possess virulence genes similar to those found in human ExPEC [27]. We measured 5 virulence genes carried by plasmids that are normally studied in human ExPEC [28, 29] and used by Johnson and collaborates [11] to distinguish avian pathogenic avian *E. coli* (APEC) from commensal *E. coli*. Our results demonstrated that strains from conventionally raised poultry have a greater number of virulence genes than the strains from free-range poultry, with the exception of the *iss* gene ($p > 0.05$). Furthermore, few strains from free-range poultry showed virulence factors, unlike strains from conventionally raised poultry, of which 75.2% had at least one virulence factor. These genes were also found in *E. coli* isolated from urinary tract infections [30], and some of these genes (*iss*, *iro*N, *omp*T, and *hly*F genes) were found also in conjugative plasmid in human *E. coli* strains isolated from sepsis, in Brazil, indicating a possible zoonotic risks [28]. According to phylogenetic classification, our results showed most prevalence of group A in strains from free-range poultry and group B1 in strains from conventionally raised poultry. Thus, the majority of the strains show characteristics relative to commensal phylogenetic groups, although most strains from conventionally raised poultry were positive for virulence factors. These results can be related to the creation system because the conventional poultries are raised in larger groups in few areas, generating a high density, which facilitates the transmission of bacteria between them because there are many virulence genes carried by plasmids, whereas free-range poultry creation is in small groups, making it more difficult to transmit pathogens [13].

Antimicrobial resistance in bacteria isolated from food of animal origin is often associated with the use of antibiotics in livestock [2, 3, 8]. Due to indiscriminate use of antimicrobials in poultry feeds, since 2006, in Europe, the use of antimicrobials as growth promoters is prohibited [31]. The use of several antibiotics including tetracyclines, β-lactams, systemic sulfonamides, and quinolones has been banned as growth promoters in many countries, for example, in Brazil [32, 33].

In the antimicrobial susceptibility test, strains isolated from conventionally raised poultry showed a higher frequency of resistance than the strains from free-range poultry to all antimicrobials. There were significant differences for the majority of the antimicrobials tested, except for cefoxitin, ceftazidime, and nitrofurantoin ($p > 0.05$). The high frequency of antimicrobial resistance in strains from conventional poultry carcasses, primarily to tetracycline, nalidixic acid, and ampicillin, can be related with the selective pressure due to the high use of antimicrobials and/or the contamination of environment in aviculture industries.

However, an interesting finding in our study was the low frequency of antimicrobial resistance in strains from free-range poultry, except to tetracycline. It is known that the use of antimicrobials in family agriculture is restricted or even absent, being casually used for treating diseases [34]. Another hypothesis for the low observed frequency is that free-range poultry normally live in small groups, compared to conventionally raised poultry, leading to individual therapeutic interventions, whereas in the poultry industry, birds are kept in larger groups, so population-based therapeutics are mostly appropriate [13].

Tetracycline was the antimicrobial with the highest frequency of resistance in both rearing systems. The high frequency may be due to the easy access to and low price of these antimicrobials and poor monitoring by regulatory bodies in veterinary medicine in Brazil because these antimicrobials have prohibited use. Another explanation of the high frequency of resistance in strains from free-range poultry is its contact with environmental microorganisms, which produce natural antibiotics, or by soil contamination with the feces of wild animals that carry antibiotic-resistant microorganisms [8, 35].

β-lactam antimicrobials, especially the third-generation cephalosporins, are the most common treatment for human infections by Enterobacteriaceae. However, a large number of resistant bacteria have emerged worldwide. Among ExPEC, β-lactamases remain the most important mechanisms of β-lactam resistance. β-lactamases are hydrolytic enzymes that cleave the β-lactam ring. The emergence of β-lactamases is mainly linked to the spread of genes encoding ESBLs and/or plasmid-mediated AmpC β-lactamases [9]. However, ESBL/AmpC-producing bacteria are now being found in increasing numbers in food-producing animals, for example, in poultry meat [5, 13, 36].

One notable finding was the presence of ESBL/AmpC β-lactamases only in strains from conventional poultry, with group 2 CTX-M and CIT groups being the most prevalent ESBL and AmpC, respectively. The absence in strains from free-range poultry may indicate the low use of antimicrobials in its production.

CTX-M-type strains are the most common ESBL type in humans, despite several reports of TEM and SHV as well [9]. Other countries have also reported a high prevalence of ESBL-producing bacteria in poultry [5, 13, 36]. In Brazil, group 2 CTX-M has been identified in *Salmonella enteric* from chickens [37].

Plasmid-mediated AmpC genes are derived from chromosomal AmpC genes, the majority of plasmid-mediated AmpC genes being found in nosocomial isolates of *E. coli*

and *Klebsiella pneumoniae*. Six families of plasmid-mediated AmpC β-lactamases have been identified [26]. Among AmpC, the CIT group was the most frequently observed in our results. Studies have related the presence of the CIT group in poultry in other countries [4, 12, 13]. In Brazil, the presence of plasmid-mediated AmpC-producing in human isolates has been sporadically reported [38, 39]. The presence of 11 AmpC-producing strains indicates the importance of studies both in human and in veterinary clinical practice.

Despite the increase of ESBL/AmpC-producing *E. coli* isolates in food-producing animals, little is known about the use of β-lactam because these are banned as growth promoters in Brazilian aviculture. One hypothesis is that the coselection and coresistance have taken place because the gene encoding ESBL and other classes of non-β-lactam can be located in the same mobile genetic element, such as plasmid or transposons [15]. In our study, ESBL/AmpC-producing strains showed resistance to one or more non-β-lactam antibiotics, mainly to tetracycline (98% of the cases). The presence of ESBL and AmpC gene was not observed in the same strain. It is possible that there is a limit to the amount of β-lactamase that a bacterial cell can accommodate and still be a viable pathogen [26].

We also note that the β-lactamases may be present in strains belonging to phylogenetic groups from commensal groups A and B1, as well as virulent strains from group D. We note also that the majority of ESBL/AmpC-producing strains have one or more virulence genes tested. This can indicate that some strains harbor antimicrobial resistance genes mediated by plasmids and perhaps are harboring virulence factors encoding genes mediated by other plasmids too. Some studies have shown that virulence plasmids and multidrug resistance plasmid were not found in the same strains [8, 40]. However, Johnson and collaborates [41] found in some APEC strains hybrid resistance plasmids encoding multiple resistance to both antimicrobials and virulence-associated genes that were able to infect human cells and cause meningitis in rats.

In our results, it is clear that even with the prohibition of many antimicrobials there is still a high frequency of antimicrobial resistance in strains from conventional poultry. The low frequency of antimicrobial resistance in strains from free-range poultry may indicate that the low use of antimicrobials in this system rearing may be related to the low frequency of resistance and virulence, which can lead to a low risk of transmission of pathogens or resistance genes to humans through consumption of chicken meat. The monitoring of antimicrobial resistance frequencies in animal foods can aid in the detection of banned poultry farming practices.

5. Conclusion

The high frequency of antimicrobial resistance, associated with several virulence factors, made *E. coli* in a potential food problem, due to the possibility of horizontal transfer of virulence genes and antimicrobial resistance to the human resident microbiota and/or human pathogens. The absence or restricted use of antimicrobials in free-range poultry production may be contributing to the lower frequency of bacterial virulence factors and resistance to antimicrobials, leading to a lower risk of their transmission to humans.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Efficient and Specific Detection of *Salmonella* in Food Samples Using a *stn*-Based Loop-Mediated Isothermal Amplification Method

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The *Salmonella* enterotoxin (*stn*) gene exhibits high homology among *S. enterica* serovars and *S. bongori*. A set of 6 specific primers targeting the *stn* gene were designed for detection of *Salmonella* spp. using the loop-mediated isothermal amplification (LAMP) method. The primers amplified target sequences in all 102 strains of 87 serovars of *Salmonella* tested and no products were detected in 57 non-*Salmonella* strains. The detection limit in pure cultures was 5 fg DNA/reaction when amplified at 65°C for 25 min. The LAMP assay could detect *Salmonella* in artificially contaminated food samples as low as 220 cells/g of food without a preenrichment step. However, the sensitivity was increased 100-fold (~2 cells/g) following 5 hr preenrichment at 35°C. The LAMP technique, with a preenrichment step for 5 and 16 hr, was shown to give 100% specificity with food samples compared to the reference culture method in which 67 out of 90 food samples gave positive results. Different food matrixes did not interfere with LAMP detection which employed a simple boiling method for DNA template preparation. The results indicate that the LAMP method, targeting the *stn* gene, has great potential for detection of *Salmonella* in food samples with both high specificity and high sensitivity.

1. Introduction

Salmonella remains a leading cause of food poisoning in humans and is also a major foodborne pathogen worldwide [1, 2]. The genus *Salmonella* is a member of the Enterobacteriaceae family and is divided into two species, *S. enterica* and *S. bongori*. More than 2500 serovars of *Salmonella*, mostly in the species of *enterica*, have been reported [3, 4]. *Salmonella* is usually transmitted to humans through consumption of contaminated food. Most often contaminated food is of animal origin (such as eggs, beef, poultry, and milk) but can also include water and vegetables [5, 6]. Due to the health risk and economic impacts of foodborne illness associated with *Salmonella*, more rapid methods with high sensitivity and specificity for *Salmonella* detection are still required.

The conventional microbiological method for the detection and identification of *Salmonella* in food samples requires multiple subculture steps, followed by biochemical and serological confirmation tests. This method is time consuming and labor intensive and typically requires 5 to 7 days depending on the biochemical test and serological confirmation utilized [7, 8]. Various molecular-based methods have been used to detect *Salmonella* and other pathogens due to their sensitivity and ability for rapid detection. Among these methods, PCR has been successfully established as a valuable method which offers the rapid, sensitive, and specific detection of the selected genes in various pathogens such as *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., and *Shigella* spp. [7, 8]. Detection of a number of foodborne pathogens, such as *L. monocytogenes*, *Salmonella* spp., and *Shigella* spp., has utilized real-time PCR allowing for the rapid analysis of samples [9–11]. Despite these advantages, PCR methods require complicated procedures and expensive

equipment, such as thermocyclers and electrophoresis units, which are not suitable for use in field conditions.

Loop-mediated isothermal amplification (LAMP) is a recently developed technique that shows promise for use under conditions in which standard laboratory equipment is not available. Advantages of the LAMP technique include ease of operation, a high degree of specificity, and rapid and simple procedures compared to PCR methods. Detection of LAMP products is also suitable to field conditions as gel electrophoresis is not required [12]. The LAMP method produces large amounts of pyrophosphate, a by-product of DNA amplification, which can easily be detected by monitoring turbidity or fluorescence [13–15]. In addition, the presence of nontarget DNA and inhibitors in the LAMP reaction has been shown to not affect the amplification results [13]. This powerful technique with reduced rates of false positives and inhibition should be a viable tool for the detection of specific pathogens in food samples, since high amounts of nontarget DNA from many food ingredients as well as several inhibitors are usually present.

The aim of this study is to develop a LAMP assay that can be applied to the *Salmonella* enterotoxin (*stn*) gene for the detection of *Salmonella* in food samples. The simple method of DNA preparation from food samples and the sensitivity and the specificity of LAMP detection procedure were also described.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions. One hundred and fifty-nine strains, including 102 strains (87 serovars) of *Salmonella enterica* and 57 non-*Salmonella* strains in the family Enterobacteriaceae, were obtained from World Health Organization National *Salmonella* and *Shigella* Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. All strains are listed in Supplementary Table S1 in Supplementary material, available online at http://dx.doi.org/10.1155/2015/356401. *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 23566 was used as the reference strain. All of bacterial cultures were stored in 15% (v/v) glycerol at −80°C. For cultivation, *Salmonella* spp. were subcultured on xylose lysine deoxycholate agar medium (XLD; Merck). Non-*Salmonella* bacterial strains were subcultured on Luria-Bertani agar (LA) plate (10 g tryptone; 5 g yeast extract; 10 g NaCl; 15 g agar; and H_2O to 1000 mL) and incubated at 37°C overnight.

2.2. Sequence Comparison of the stn Gene. The *stn* gene sequences from *Salmonella* and non-*Salmonella* species of the Enterobacteriaceae family were obtained from the GenBank database. Pairwise sequence comparisons were performed using the EMBOSS Needle tool program (EMBL-EBI). Sequences were aligned using the MUSCLE multisequence alignment program. Phylogenetic relationships and evolutionary history were inferred from this alignment using the MEGA5 software program and the Neighbor-Joining method, respectively. The bootstrap consensus tree from 2000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Table 1: Oligonucleotide primer sequences used in *stn* LAMP analysis.

Primer	Primer type	Sequence (5′-3′)
F3	Forward outer (F3)	5′ ACCAGATTCAGGGAGTGAGT 3′
B3	Backward outer (B3)	5′ CGCGCACGAAATTCGTAAC 3′
FIP	Forward-inner (F1c-F2)	5′ ACCGGGTGGTAAGCGAATTGC-GAGGTTAACCGTCTGGAGC 3′
BIP	Backward-inner (B1c-B2)	5′ TCGGCCTCTTTGGCCATCAC-TGGCGAAATACTTTGCCGAG 3′
Loop F	Loop forward (LF)	5′ TGGTAAAGCCCGCGCATCTG 3′
Loop B	Loop backward (LB)	5′ GCGCCAGTTCATGCGACTCG 3′

Note: primers for LAMP were designed using the *stn* gene (GenBank Accession number L16014) from *Salmonella enterica* subsp. *enterica* serovar Typhimurium.

2.3. Primer Design for LAMP. A set of four primers comprising two inner primers (FIP and BIP) and two outer primers (F3 and B3) that recognize six distinct sequences within the *Salmonella* enterotoxin (*stn*) gene of *Salmonella* Typhimurium (GenBank Accession Number L16014) were designed. To compare amplification efficiency, loop primers, LF and LB (between the F1/F2 region and B1/B2 region, resp.), were also designed to check their role in increasing the sensitivity of detection. A set of LAMP primers was designed using the PrimerExplorer V4, LAMP primer design program (http://primerexplorer.jp/e/index.html). The designed primers were compared with the NCBI sequence database to confirm the specificity of the primers. The nucleotide sequences and annealing portions of each primer are shown in Table 1 and Supplementary Figure S1, respectively.

2.4. LAMP Reaction and LAMP Product Detection. The LAMP reaction was performed in a total volume of 25 μL containing the following components (final concentration): 1.6 μM each of FIP and BIP primers, 0.2 μM each of F3 and B3 primers, 0.8 μM each of LF and LB primers (in the same LAMP reaction), 1.6 mM of deoxyribonucleotide triphosphate mixture (dNTPs), 1 M betaine (Sigma, B2629, St. Louis, USA), 6 mM $MgSO_4$, 1x thermopol buffer (New England Biolabs, B9004S, Beverly, USA), 1 μL (8 U) of *Bst* DNA polymerase large fragment (New England Biolabs, M0275S, Beverly, USA), and 5 μL of DNA template solution. The reaction temperature was optimized by incubating the reaction mixture under isothermal conditions between 60 and 70°C for 60 min. The reaction time was optimized by varying the time in each reaction from 10 to 60 min, at 5 min intervals in each condition at the optimal temperature (65°C). The reaction was terminated by heating at 80°C for 5 min. *S.* Typhimurium ATCC 23566 cells or its isolated DNA (1 ng/reaction) was used as positive controls. LF and LB primers were added to determine if these primers increased amplification efficacy. For analysis of the LAMP

Table 2: The *stn* gene sequence similarity values among 77 bacterial strains of Enterobacteriaceae.

Genera	Similarity to					
	Klebsiella	*Escherichia*	*Shigella*	*Enterobacter*	*Citrobacter*	*Salmonella enterica*
Klebsiella (2)	71.6–100					
Escherichia (49)[a]	68.3–73.7	89–100				
Shigella (8)	67.1–73.0	91.5–98.0	94.9–100			
Enterobacter (1)	64.8–67.0	68.1–68.8	67.5–69.2	100		
Citrobacter (1)	60.0–63.9	63.4–64.2	63.5–65.2	70.1	100	
Salmonella enterica (16)	57.0–60.4	61.6–66.7	63.0–64.5	66.9–67.8	73.2–74.0	96.9–100
Salmonella bongori (1)	57.6–60.4	60.0–66.2	62.6–64.5	67.8	73.9	83.8–84.7

[a]The number in parenthesis is the number of sequences collected from the NCBI database.

products, the turbidity resulting from the white precipitate of magnesium pyrophosphate in the mixture was observed by eye and confirmed by monitoring the formation of a green color under normal light following addition of SYBR green. The negative reaction was orange in color. LAMP products (2.5 μL) were also analyzed by electrophoresis using 2% agarose gel, stained with ethidium bromide, and visualized using a UV transilluminator.

2.5. DNA Template Preparation and Sensitivity of LAMP Detection. DNA template for all bacterial strains was prepared using the boiling method [16]. *Salmonella* DNA templates were prepared from 1.5 mL of overnight cultures grown in lactose broth incubated at 37°C. Cells were harvested and washed twice and then resuspended in 100 μL of TE buffer. Lysis was performed by boiling at 100°C for 10 min, and supernatant was collected. Non-*Salmonella* strains were grown in LB broth and DNA templates were prepared as described above. To determine the minimum DNA concentration required for the LAMP reaction template DNA from *S.* Typhimurium ATCC 23566 was prepared following the total DNA isolation procedure as described by Wilson [17]. Genomic DNA at 15.625 pg, 3.125 pg, 625 fg, 125 fg, 25 fg, 5 fg, and 1 fg DNA/reaction was used as DNA templates in a total volume of 25 μL. To determine the minimal cell number, *S.* Typhimurium ATCC 23566 was cultured in lactose broth and incubated at 37°C overnight (16 hr), then subcultured (1% inoculum) in freshly prepared lactose broth, and further incubated at 35°C for 5 hr with shaking. LAMP assays were performed using cell numbers ranging from 0 to 1000 cells/reaction and DNA template was prepared by the boiling method as described above. The numbers of cells were monitored by the plate count technique on XLD agar plate after incubation at 37°C for 48 hr.

2.6. Salmonella Detection in Artificially Contaminated Food. A single colony of *S.* Typhimurium ATCC 23566 was picked from XLD agar plate and was inoculated into lactose broth and incubated at 37°C overnight (16 hr). The number of viable cells was obtained using the plate count technique on XLD agar plate with incubation at 37°C for 48 hr. The plate count was performed in triplicate. Minced pork meat was decontaminated by autoclaving at 121°C for use as a sterile food sample [18]. The autoclaved food sample (25 g in 225 mL of lactose broth) was artificially contaminated by spiking with 1000 μL of appropriate dilutions of *S.* Typhimurium ATCC 23566 to achieve 5.5×10^6–55 cells/25 g of food sample. The inoculated food sample was then mixed with 225 mL lactose broth and enriched at 35°C for 5 hr. 1.5 mL of inoculated food sample was taken out at 0 and 5 hr time points and stood without shaking for 5 min to allow particulate matter to settle. One mL of the upper portion was collected and centrifuged for 5 min at 10000 rpm. The pellet was washed twice with 500 μL of TE buffer and resuspended in 100 μL of TE buffer. DNA was extracted using the simple boiling method as described above. The supernatant (5 μL) was also directly used as the DNA template for LAMP amplification. These experiments were performed in triplicate. Autoclaved minced pork meat without *Salmonella* inoculation was included in every experiment as a negative control.

2.7. Detection of Salmonella in Naturally Contaminated Food. To determine the validity and reliability of LAMP detection of the *stn* gene for *Salmonella* identification in food samples a comparative study between LAMP and the reference culture method was performed. Various kinds of foods with possible naturally occurring *Salmonella* contamination were investigated. A total of 90 food samples, 30 each of minced pork meat, chicken meat, and fresh vegetables, were purchased randomly in a local market in Bangkok. All food samples were transported to the laboratory in an ice box and were examined immediately after purchase. Each food sample (25 g) was homogenized in 225 mL of lactose broth. The mixture was incubated at 35°C and 1.5 mL was collected for DNA extraction after 5 and 16 hr of incubation. DNA was then prepared from food samples for use in LAMP detection. The reference culture method was performed in parallel as described in the *Bacteriological Analytical Manual* (*BAM 8th Edition*) [19]. The performance indicators for qualitative methods and efficacy of LAMP method in comparison with the reference culture method for *Salmonella* detection in food samples were calculated using the method of Galen [20].

3. Results

3.1. stn Gene Sequence Data Analysis. A total of 77 putative *stn* whole gene sequences from Enterobacteriaceae strains were collected from GenBank. Similarities of *stn* among various genera are listed in Table 2. The nucleotide sequence

Table 3: Optimization of LAMP assay for *Salmonella* detection by targeting the *stn* gene.

Conditions	The tested range	Difference	Good yield	Selected condition[a]
Temperature	60–65°C	1°C	64–66°C	65°C
Time	0–60 min	5 min	15–60 min	25 min
Loop primers	0.5–1.0 μM	0.1 μM	0.8–1.0 μM	0.8 μM
Outer primers	0.1–0.5 μM	0.1 μM	0.2–0.5 μM	0.2 μM
$MgSO_4$	4–8 mM	0.5 mM	6–7.5 mM	7 mM
dNTPs	0.8–2.4 mM	0.2 mM	1.2–1.8 mM	1.6 mM
Betaine	0.5–1.5 M	0.1 M	0.6–1.5 M	1.0 M
Bst DNA polymerase	4–12 units	1 unit	8–12 units	8 units

[a]The final concentrations of reagents or temperature and times used in LAMP analysis.
The concentration of FIP and BIP primers are fixed at 1.6 μM.

of *stn* among 16 *Salmonella enterica* strains shows 96.9–100% homology. Only one *stn* gene sequence for *S. bongori* was available which exhibited 83.8–84.7% homology to the 16 *stn* sequences from *S. enterica* (Table 2). The *stn* sequence from *Salmonella* exhibited 57.0% to 74.0% sequence homology to *stn* genes from other genera, with the highest homology to that of *Citrobacter* at 73.2% to 74.0%. The phylogenetic relationships of *stn* from 12 serovars, 17 strains (16 for *S. enterica* and one for *S. bongori*) in the genus *Salmonella* with other genera in the family Enterobacteriaceae were determined. The dendrograms inferred from the *stn* sequence alignment using the Neighbor-Joining method (Supplementary Figure S2) and Maximum Likelihood method yielded similar topologies in which *Salmonella* formed individual clusters. The toxin gene of *S. aureus* was used as the out group. The data suggested that the *stn* gene should provide a genus-specific target sequence for detection of *Salmonella.*

3.2. Optimal Condition for the LAMP. The optimal temperature for LAMP assay was determined by varying the temperature between 60 and 70°C for 60 min in 1°C increments using purified DNA template. The LAMP products amplified between 64 and 66°C showed clear and distinct DNA bands with higher density compared to other samples. Therefore, 65°C was set as an optimal temperature. Reaction times, 5–60 min at 5 min intervals, were analyzed with the reaction temperature at 65°C using 0.8 μM each of LF and LB loop primers. LAMP products were detected as early at 15 min (Supplementary Figure S3(B), lane 3) but the clear and intense bands were observed at 20 min (Supplementary Figure S3(B), lane 4). In the absence of loop primers clear and dense bands were observed after 35 min (Supplementary Figure S3(A), lane 7). Therefore, the optimal condition for the *stn* LAMP assay was set at 65°C for 25 min with 0.8 μM each of loop primers, 1.6 μM each of FIP and BIP primers, and 0.2 μM each of outer primers. The effect of various LAMP reagent concentrations ($MgCl_2$, dNTPs, betaine, and *Bst* DNA polymerase) was also studied (data not shown). The optimal conditions for the highest sensitivity for *Salmonella stn* gene detection using LAMP are summarized in Table 3.

3.3. Specificity of LAMP. The results for the specificity test of the *stn* LAMP assay are shown in Supplementary Table S1. In this analysis all of 102 strains of 87 serovars of *Salmonella* gave positive results. In contrast, the 57 non-*Salmonella* strains in family Enterobacteriaceae including 6 strains of *Citrobacter* examined by LAMP were negative. Product formation in the LAMP assays was monitored by observing the presence of white turbidity and by observing green color following addition of SYBR green in the reaction mixture. The presence of amplified products was also confirmed using gel electrophoresis with 2% agarose gels. No amplified products were observed in LAMP reactions lacking *Salmonella* DNA demonstrating that amplification of the *stn* gene was highly specific for *Salmonella* detection. Examples of LAMP assays using DNA template from some *Salmonella* serovars and other enteric bacteria are shown in Supplementary Figure S4.

3.4. Sensitivity of Detection. Analysis using purified DNA prepared from *S.* Typhimurium ATCC 23566 revealed that the lowest DNA concentration that could promote *stn* amplification by LAMP (at 65°C, 25 min with loop primers) was 5 fg DNA/25 μL reaction (Supplementary Figure S5(A)). Similar analysis using DNA prepared from whole cells by boiling method indicates that a minimum of 1 cell/reaction was sufficient to give a positive result with the *stn* LAMP assay under these conditions (Supplementary Figure S5(B)).

3.5. Detection of Salmonella in Artificially and Naturally Contaminated Food Samples. All six samples of autoclaved minced pork meat (25 g) in 225 mL of lactose broth inoculated with 5.5×10^6–55 cells of *S.* Typhimurium ATCC 23566 gave positive result. In contrast, uninoculated meat samples were negative. The detection limit of *Salmonella* in artificially contaminated food using the LAMP assay was 5.5×10^3 cells/250 mL (220 cells/g of food sample) without an enrichment step (at 0 hr), as shown in Table 4. The sensitivity increased to 55 cells/250 mL (2 cells/g of food sample) after incubation of the inoculated food sample for 5 hr at 35°C prior to LAMP analysis (Table 4). A total of 90 food samples were analyzed using both the reference culture method according to BAM and the LAMP method. This analysis used 30 samples each of minced pork meat, chicken meat, and fresh vegetables enriched for either 5 or 16 hr. The results (data not shown) indicated that 67 samples were positive and 23 samples were negative by both methods after enrichment for 5 hr.

Table 4: Sensitivity for LAMP detection of *Salmonella* in artificially contaminated food samples using DNA templates prepared by the boiling method.

Total cells per[a]				Enrichment time	
250 mL of food suspension	Gram of food sample	1 mL of food suspension[b]	LAMP reaction[c]	(0 hr)	(5 hr)
5.5×10^6	2.2×10^5	2.2×10^4	1.1×10^3	+	+
5.5×10^5	2.2×10^4	2.2×10^3	1.1×10^2	+	+
5.5×10^4	2.2×10^3	2.2×10^2	1.1×10^1	+	+
5.5×10^3	2.2×10^2	2.2×10	1.1	+	+
5.5×10^2	2.2×10	2.2	0	−	+
5.5×10^1	2.2	0.2	0	−	+

[a]The number of cells at 0 hr (before enrichment) using 25 g of food sample in 225 mL of lactose broth.
[b]One mL of food sample was used to prepare DNA template by the boiling method. Samples were resuspended in 100 μL of TE buffer and used as DNA template.
[c]5 μL of DNA template was used in a 25 μL LAMP reaction. Loop primers were included in the reaction mixture.

The meat samples gave higher positive results (28 and 25 for minced pork meat and chicken meat, resp.) than those of fresh vegetable (14 out of 30 samples) (data not shown). Preenrichment times of 5 hr and 16 hr gave similar results (data not shown). The overall relative diagnostic specificity and accuracy were judged to be 100% as no false-negative or false-positive results were obtained from the LAMP assay compared to the culture method.

4. Discussion

The LAMP method has been utilized to efficiently detect several foodborne pathogens such as *Staphylococcus aureus* [21], *Bacillus cereus* [22], *Vibrio vulnificus* [23], and *Salmonella*. Previous reports for LAMP detection of *Salmonella* utilized several gene targets including *invA* [14, 16, 24, 25], *phoP* [15], *Sdf I* [12], and *IS200/IS1351* [26]. More recently, a *fimY*-based LAMP assay has been used to detect 80 *Salmonella* strains of 24 serovars [27]. In our study, we targeted the *stn* (*Salmonella* enterotoxin gene) which is widely distributed among *Salmonella* serovars and has been identified in all 95 strains of *Salmonella* [28]. The *stn* gene exhibits high nucleotide sequence homology among *Salmonella* strains but low homology to the corresponding gene among other closely related enteric bacteria. It has also been reported that *stn* gene amplification can be effectively used to specifically detect 52 strains of *S. enterica* and 2 strains of *S. bongori* without cross-reacting with several common intestinal strains [29]. Based on our results and previous findings it can be established that *stn* is a suitable target gene for direct detection of *Salmonella* in biological samples.

The *stn* gene from *S. enterica* encodes a protein of approximately 29 kDa, utilizing a TTG start codon, and contains a portion of the conserved motif found in other protein toxins with ADP ribosylation activity [30]. The results of LAMP assay using the *stn* gene in our study confirmed that it was an appropriate target to specifically detect *Salmonella* strains without cross-reaction with other closely related enteric bacteria from a complex matrix, such as food samples. We did not include *S. bongori* in our analysis; however, *S. bongori* displays 88% sequence identity with *S. enterica stn*. Primers targeting the *stn* gene efficiently amplify both *S. enterica* and *S. bongori* in real-time PCR [31] and PCR [29] analysis, suggesting that the LAMP procedure should also be applicable for detection of *S. bongori*. The available *stn* gene sequences of *Enterobacter* and *Citrobacter* strains in GenBank database, respectively, showed 66.9–67.8% and 73.2–74.0% similarity with *stn* gene sequence from 16 *Salmonella* strains. However, when 5 strains of *Enterobacter* and 6 strains of *Citrobacter* were used in the *stn* LAMP test with *Salmonella* specific primers, no positive results were detected. The lack of false-positive results with non-*Salmonella* strains suggests that *stn* can be utilized as specific target gene for *Salmonella* detection.

The presence of loop primers in reaction mixtures reduced the time required for amplification of *stn* gene to 25 min and provided high sensitivity, allowing the detection of only one *Salmonella* cell in artificially contaminated food samples. The LAMP assay targeting *invA* gene (also with loop primers) can detect pure culture of *Salmonella* at 2.8 cells/reaction tube [14]. In other studies, the LAMP method targeting *phoP* gene for detecting *Salmonella* in 20 hr preenrichment food samples shows the lowest limit of detection at 35 cells/reaction tube. Hence, our optimized LAMP method targeting *stn* gene provided more sensitive detection limit compared to *invA* and *phoP* genes. The gene sequence similarities of *phoP* [15] between different genera of enteric bacteria compared to *Salmonella* are quite high at 70.8–85.9%, whereas, for *stn*, sequence similarities among these bacteria were only 57.6–73.9% (Table 4). In both studies on *phoP* and *stn*, *Citrobacter* is the genus most similar to *Salmonella*, showing 85.6–85.9% and 73.9% homology for *phoP* and *stn* genes, respectively. The low sequence similarity of *stn* genes among other enteric bacteria compared to *Salmonella* may provide more specific detection of *Salmonella* in different kinds of food samples in which various enteric bacteria are simultaneously present. Therefore, these assay conditions targeting the *stn* gene constitute a valuable procedure for the rapid detection of *Salmonella* in food samples.

5. Conclusion

Salmonella enterotoxin (*stn*) gene is highly conserved among *S. enterica* serovars and *S. bongori* [28, 29, 31–33]. The six primers designed from *stn* gene could specifically detect 87 serovars of *S. enterica* (102 strains) without cross-reacting

with 57 non-*Salmonella* strains, including *C. diversus* (3 strains) and *C. freundii* (3 strains).

The *stn* amplification using the LAMP procedure was shown to be highly accurate for detection of *Salmonella* in various food matrices without cross-reacting with other contaminated bacteria in the food samples, even from other closely related enteric bacteria. This LAMP assay using *stn* as a target gene has the potential as a rapid method for detection of *Salmonella* with high sensitivity and specificity and could be used as a method of choice in diagnostic food laboratories.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Contribution of Avian *Salmonella enterica* Isolates to Human Salmonellosis Cases in Constantine (Algeria)

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An epidemiological investigation was carried out on one hundred *Salmonella* isolates from broiler farms, slaughterhouses, and human patients in the Constantine region of Algeria, in order to explore the contribution of avian strains to human salmonellosis cases in this region over the same period of time. The isolates were characterized by phenotypic as well as genotypic methods. A large variety of antimicrobial resistance profiles was found among human isolates, while only seven profiles were found among avian isolates. Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), Insertion Sequence *200*-PCR (IS*200*-PCR), and Pulsed Field Gel Electrophoresis (PFGE) resulted in the allocation of the isolates to 16, 20, and 34 different profiles, respectively. The 3 genotyping methods led to complementary results by underlining the clonality of some serovars with the diffusion and persistence of a single clone in the Constantine area as well as stressing the polymorphism present in isolates belonging to other serovars, indicating the diversity of potential reservoirs of nontyphoidal *Salmonella*. Altogether, our results seem to indicate that nontyphoidal avian *Salmonella* may play an important role in human salmonellosis in the Constantine region.

1. Introduction

Salmonella remains a major cause of illness in both humans and animals worldwide [1, 2]. It is estimated that *Salmonella* spp. are responsible for 93.8 million cases of human gastroenteritis and 155,000 deaths worldwide each year [3]. In the European Union, over 100,000 cases of salmonellosis were reported to EnterNet in 2003 [4] and over 90,000 cases in 2012, even though human salmonellosis cases have decreased regularly since 2005 [5]. It should be stressed that the observed reduction in salmonellosis cases is presumably the result of successful *Salmonella* control programmes in poultry populations [5]. *Salmonella* is also a major public health concern in developing countries [6–8].

Salmonellosis due to nontyphoidal *Salmonella* is mainly associated with eating contaminated eggs, poultry meat, and pork. Contaminated poultry meat is identified as one of the principal sources of *Salmonella* in humans [2, 9]. Furthermore, one of the most frequent causes of infection by *Salmonella* reported in humans is the handling of raw poultry carcasses and products, together with the consumption of undercooked poultry meat [10].

The contamination of food products with *Salmonella* generates serious consequences for public health and the

economy. This has motivated numerous studies designed to investigate the survival capacity of this bacterium and its transmission routes in farm-animals and their environment [11].

In the Constantine region (Algeria), a recent study showed that 37% of broiler farms and 53% of slaughterhouses were positive for *Salmonella* [12], with a predominance of *S*. Hadar, *S*. Virchow, *S*. Infantis, *S*. Albany, and *S*. Typhimurium. In a nearby region, 44% of laying hen flocks were reported to be positive for *Salmonella* [13].

In this study, we report on the epidemiological investigation of a certain number of serovars, isolated from broiler breeding farms, slaughterhouses, and human patients within the Constantine region.

Combined phenotypic and genotypic methods were used to assess the relationships between *Salmonella* strains isolated from these sources, in order to evaluate the contribution of avian strains to human salmonellosis in the region during the 2-year study. Phenotypic methods consisted of serotyping and antimicrobial susceptibility testing, whereas genotypic techniques were based on polymerase chain reaction (PCR) (i.e., Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Insertion Sequence *200*-PCR (IS*200*-PCR)) and macrorestriction (i.e., Pulsed Field Gel Electrophoresis (PFGE)).

2. Materials and Methods

2.1. Bacterial Strains. For this study, we analysed 100 isolates recovered over a 2-year period (2006 through 2007) in the Constantine region (Table 1). The 45 human isolates studied (named H1 to H45) were obtained from the Constantine Hospital, whereas the 55 isolates of avian origin (named A1 to A55) were collected from poultry farms and slaughterhouses.

The isolation of avian strains was performed according to the NF U47-100 and NF U47-101 procedures [14, 15] at the Food Hygiene Laboratory from the Constantine Veterinary Sciences Department. Serotyping was carried out according to the White-Kauffmann-Le Minor scheme [16], as previously described [12].

2.2. Bacterial Susceptibility to Antibiotics. The antimicrobial susceptibility tests were performed using the disk diffusion method and interpreted as recommended by the "Comité de l'Antibiogramme de la Société Française de Microbiologie" [17]. Antimicrobials tested (load, breakpoints (mm)) were ampicillin (10 μg, 19–14), amoxicillin-clavulanic acid (20/10 μg, 21–14), cephalothin (30 μg, 18–12), cefotaxime (30 μg, 21–15), ceftazidime (30 μg, 21–15), streptomycin (10 IU, 15–13), gentamicin (10 IU, 16–14), kanamycin (30 IU, 17–15), chloramphenicol (30 μg, 23–19), tetracycline (30 IU, 19–17), sulphamethoxazole-trimethoprim (23.75 μg + 1.25 μg, 16–10), sulphonamides (200 μg, 17–12), nalidixic acid (30 μg, 20–15), ofloxacin (5 μg, 22–16), enrofloxacin (5 μg, 22–17), and colistin (50 μg, 15). Zone diameters were read using the automated scanner Osiris (Bio-Rad).

2.3. PCR Methods. DNA was extracted by a boiling method as described previously [18]. The intergenic segments were amplified using the primers' sequences described by Millemann et al. [18] and Versalovic et al. [19]. All amplifications were performed on a Perkin Elmer 9700 thermal cycler (Courtaboeuf, France) as previously described [18].

2.4. PFGE Genotyping. PFGE was performed using a CHEF-DR III system (Bio-Rad, Marnes La-Coquette, France) according to the Salm-gene and PulseNet standardized protocol [20–22]. Two endonucleases were used, *Xba*I for all serovars and *Bln*I for *S*. Hadar. The *S. enterica* Braenderup H9812 strain was used as an internal control and molecular size marker [23]. DNA patterns were analysed with BioNumerics software (V 6.6, Applied Maths, Sint-Martens-Latem, Belgium). Dendrograms were produced using the Dice coefficient and an unweighted pair group method with arithmetic averages (UPGMA) with a 1% tolerance limit and 1% optimization (Pulsenet Europe recommendation [20]).

3. Results

Salmonella isolates were grouped into 16 different serovars (Table 1). Six serovars, namely, Agona, Anatum, Blockley, Indiana, Kentucky, and Senftenberg, were only recovered from humans during the two-year study, whereas 3 serovars, namely, Carnac, Montevideo, and Rissen, were only isolated from poultry. Isolates belonging to the 7 remaining serovars, that is, Albany, Enteritidis, Hadar, Heidelberg, Infantis, Typhimurium, and Virchow, were recovered from both poultry and humans.

A total of 16 ERIC-PCR, 20 IS*200*-PCR, 30 antimicrobial resistance, and 34 PFGE profiles were generated from the 100 isolates. For all isolates studied, those belonging to the same serovar clustered together (Table 1 and Figure 1).

The different ERIC-PCR profiles obtained were numbered from I to XVI and IS-PCR profiles were identified by the letters A through T (Table 1). Rissen and Infantis isolates shared the same ERIC- and IS*200*-PCR profiles.

The 34 different PFGE profiles obtained were numbered according to the preexisting database. Based on PFGE patterns, different situations were established among the analysed isolates, which led to various hypotheses. All PFGE results are shown in Table 1 and Figure 1.

4. Discussion

Evaluating the contribution of various animal sources to the burden of human salmonellosis is very difficult and requires microbial subtyping approaches [24] that depend on the comparison of the phenotypic and genotypic characteristics of the isolates studied. This consists of comparing serovars isolated from animals and humans to normal findings in both national and international serovar-based surveillance databases. Finally, the use of molecular markers for which there is a database may be also useful.

Table 1: Characteristics of the avian and human studied isolates.

Strains	Serovars	ERIC-PCR	IS-PCR	Antimicrobial resistance pattern*	PFGE profile	Sources
H17	*S.* Agona	I	A	AM, CEF, CTX, SXT, SSS, GEN, STR	SAGOXB0004	Human
H45	*S.* Albany	II	B	NAL, OFX, STR	SABYXB0003	Human
A81, A88, A89				NAL, OFX, STR	SABYXB0003	Farm
A80				ENR, NAL, OFX, STR	SABYXB0003	Farm
A85, A86		III	C	NAL, OFX, STR	SABYXB0003	Slaughter
H38	*S.* Anatum	IV	D	Susceptible	SANAXB0013	Human
H21	*S.* Blockley	V	E	NAL	SBLOXB0001	Human
H41				Susceptible	SBLOXB0001	Human
A78, A79, A90, A91	*S.* Carnac	VI	F	Susceptible	SCARXB0001	Farm
H9	*S.* Enteritidis	VII	G	AMP, CAZ, SSS, TET	SENTXB0026	Human
H11				Susceptible	SENTXB0026	Human
H3, H14, H31, H32, H47				NAL, OFX	SENTXB0001	Human
H10				AMP, CTX, SSS, TET	SENTXB0001	Human
H25				NAL	SENTXB0001	Human
H26				NAL	SENTXB0013	Human
A82, A87				Susceptible	SENTXB0016	Human
H1				AMP, NAL	SENTXB0035	Human
H24				NAL, OFX	SENTXB0032	Slaughter
H2				NAL	SENTXB0033	Human
H7			H	NAL, OFX	SENTXB0001	Human
H4	*S.* Hadar	VIII	I	ENR, NAL, OFX, STR, TET	SHADXB0003	Human
H5				KAN, NAL, OFX, STR, TET	SHADXB0003	Human
H18, H44				AMP, NAL, OFX, STR, TET	SHADXB0003	Human
A28, A29, A30, A31, A32, A56				STR, TET	SHADXB0003	Slaughter
A36, A37, A38, A39				STR, TET	SHADXB0003	Farm
A33, A34, A35, A40,				STR, TET	SHADXB0003	Farm
A41, A42, A43, A44				STR, TET	SHADXB0003	Farm
A26, A27			J	STR, TET	SHADXB0003	Slaughter
H13	*S.* Heidelberg	IX	K	NAL, OFX	SHIDXB0002	Human
A60				NAL, OFX, STR	SHIDXB0002	Farm
H17				NAL	SHIDXB0009	Human
H23				NAL, OFX	SHIDXB0010	Human
H33				NAL, OFX	SHIDXB0001	Human
H46	*S.* Indiana	X	L	Susceptible	SINDXB0005	Human
A22, A23, A24, A25	*S.* Infantis	XI	M	NAL	SINFXB0001	Farm
A48, A49				Susceptible	SINFXB0001	Farm
H12				Susceptible	SINFXB0005	Human
H27, H28	*S.* Kentucky	XII	N	AMP, CAZ, CEF, CTX, GEN, KAN, SSS, SXT	SKNTXB0006	Human
A67	*S.* Montevideo	XIII	O	Susceptible	SMVDXB005	Slaughter
A21	*S.* Rissen	XI	P	NAL	—	Farm

Table 1: Continued.

Strains	Serovars	ERIC-PCR	IS-PCR	Antimicrobial resistance pattern*	PFGE profile	Sources
H34	*S.* Senftenberg	XIV	Q	NAL, STR	SSFTXB0039	Human
H15				AM, CAZ, CF, CTX, GM, K, NAL, S, SSS	SSFTXB0013	Human
H30				NAL, STR	SSFTXB0038	Human
H35, H16			R	NAL, STR	SSFTXB0037	Human
H37				NAL, STR	SSFTXB0040	Human
H29	*S.* Typhimurium	XV	S	NAL	STYMXB0093	Human
H19, H20, H22, H36				AMP, CHL, SSS, STR, TET	STYMXB0035	Human
H8				NAL	STYMXB0089	Human
H6				NAL	STYMXB0005	Human
A17, A18, A45, A46			T	STR	STYMXB0021	Slaughter
A19				NAL, STR	STYMXB0021	Slaughter
A63	*S.* Virchow	XVI	U	Susceptible	SVIRXB0017	Farm
A66				NAL	SVIRXB0017	Farm
H40				Susceptible	SVIRXB0005	Human
A20				NAL	SVIRXB0005	Slaughter
A53, A65				Susceptible	SVIRXB0005	Slaughter
A62, A64, A77, A92				Susceptible	SVIRXB0005	Farm

*Susceptible: susceptible to all tested antibiotics. AMP: ampicillin; AMC: amoxicillin-clavulanic acid; CAZ: ceftazidime; CEF: cephalothin; CHL: chloramphenicol; CST: colistin; CTX: cefotaxime; ENR: enrofloxacin; GEN: gentamicin; K: kanamycin; NAL: nalidixic acid; OFX: ofloxacin; SSS: sulphonamides; STR: streptomycin; SXT: trimethoprim-sulfamethoxazole; TET: tetracycline.

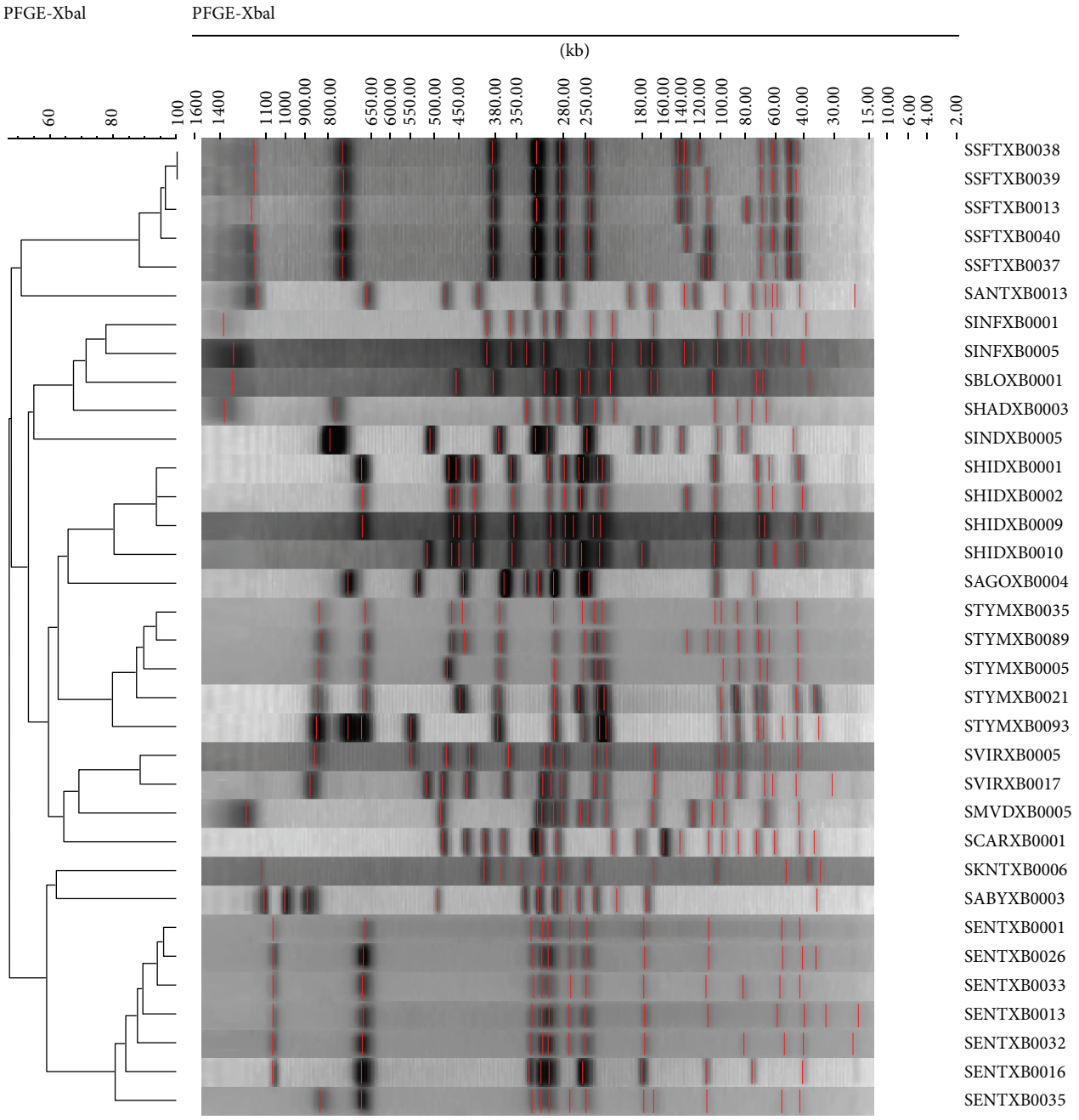

Figure 1: Dendrogram based on *Xba*I Pulsed Field Gel Electrophoresis (PFGE) profiles of major *Salmonella* serovars identified in Constantine (Algeria). Similarity percentages are figured on the left; names of the profiles are listed on the right. SABY: *Salmonella* Albany; SANT: *Salmonella* Anatum; SAGO: *Salmonella* Agona; SBLO: *Salmonella* Blockley; SCAR: *Salmonella* Carnac; SENT: *Salmonella* Enteritidis; SHAD: *Salmonella* Hadar; SHID: *Salmonella* Heidelberg; SIND: *Salmonella* Indiana; SINF: *Salmonella* Infantis; SKNT: *Salmonella* Kentucky; SMVD: *Salmonella* Montevideo; SSFT: *Salmonella* Senftenberg; STYM: *Salmonella* Typhimurium; SVIR: *Salmonella* Virchow.

4.1. Serovars Isolated in Poultry and Humans. The serovars isolated from broilers in our study represent those usually present in broilers worldwide, especially in the USA and Europe [5, 25]. In our study, we recovered 6 serovars from broilers (i.e., on farms or in slaughterhouses) that are among the top 10 serovars encountered in Europe, including Enteritidis, Hadar, Indiana, Infantis, Typhimurium, and Virchow [5]. However, our study did not reflect this order as Hadar was isolated most frequently followed by Virchow, Infantis, and Albany.

Enteritidis and Typhimurium were the serovars most often isolated from human clinical cases in this study. This is generally consistent with other worldwide studies, for instance, in the USA and in Europe, as well as in Africa [5, 6, 25]. Senftenberg was ranked third, followed by Heidelberg, Blockley, and Kentucky. The high occurrence of Senftenberg

is somewhat surprising and may be related to extensive commercial links with France. On the other hand one would have expected a slightly higher number of *S*. Kentucky isolates due to the recent emergence and distribution of this serovar in Africa [26, 27].

Interestingly, although it is rarely isolated from broiler and laying hen flocks, Albany was frequently isolated from broilers in our study [13, 26, 28]. Carnac is an extremely rare serovar in both poultry and humans. For instance, only one Carnac isolate was recovered from poultry in the European base line studies in 2008 [27] and the 2013 USA atlas for *Salmonella* did not report Carnac isolates for humans [29].

Some serovars (i.e., Agona, Anatum, Blockley, Indiana, Kentucky, and especially Senftenberg) were only isolated from humans in our study. However, those serovars are frequently isolated from various poultry species and are associated with chicken consumption when isolated in humans [28]. Senftenberg is mainly isolated in hatcheries and laying hen farms, and, in 2012, it ranked fourth among laboratory-confirmed *Salmonella* isolates from nonclinical nonhuman sources submitted to the National Veterinary Services Laboratories (NVSL) for typing in the USA [24]. This is one of the most commonly isolated serovars in France. For instance, in 2008, *S*. Senftenberg ranked first in total isolates collected from nonhuman sources as well as from poultry farm environments [28]. Kentucky is an emerging serovar in poultry and human and, recently, a particular multidrug resistant (MDR) phenotype has emerged in Africa and spread throughout poultry plants [30]. This MDR phenotype has also been isolated from laying hen flocks in Algeria [13]. Nevertheless, the Kentucky isolates from this study, although they were multidrug resistant, could not be linked to the global epidemic described by le Hello et al. [30] as these isolates are fully susceptible to fluoroquinolones.

Thus, considering the 7 serovars isolated in this study from both humans and poultry as well as the 6 serovars usually linked to human infection by poultry, isolates belonging to 13 of the 16 identified serovars suggest the potential link between poultry contamination and human salmonellosis.

4.2. Contribution of Epidemiological Markers to the Comparison of Avian Isolates and Human Isolates. Among the 7 serovars isolated from both humans and poultry in this study, 4 serovars (i.e., Albany, Hadar, Heidelberg, and Virchow) included human and avian isolates with indistinguishable patterns. In contrast, human and avian strain patterns did not match for serovars Enteritidis, Infantis, or Typhimurium.

4.2.1. Matching Avian and Human Patterns. Serovar Albany strains were isolated from 3 different sources (i.e., humans, breeding farms, and slaughterhouses) but could not be differentiated by PFGE after digestion by restriction enzyme *Xba*I. There were only two strains of this serovar in the ANSES database and the identified profile SABYXB0003 was new. Therefore, it remains difficult to determine any genetic heterogeneity among these isolates. However, the two isolates from the slaughterhouses shared distinct ERIC-PCR and IS-PCR profiles. As a whole, our results suggest an epidemiological link between strains isolated from breeding farms, humans and, to a lesser extent, slaughterhouses. This conclusion is supported by the very similar antimicrobial resistance patterns observed, especially since fluoroquinolones were targeted.

Twenty-four Hadar isolates isolated from slaughterhouses, farms, and humans were characterized. All isolates merged with a single PFGE profile, with digestion by either *Xba*I or *Bln*I restriction enzymes, which seems to demonstrate the clonal character of the strains isolated from broiler chickens and humans. Nevertheless, we must be cautious since Hadar is considered to be a genetically homogeneous serovar (DI = 0.70 [20]). The comparison with the ANSES database showed that, with *Xba*I, 24 profiles had been identified out of the 153 strains of this previously studied serovar and the DI was only 0.48. This possible epidemiological link also seems to be supported by the single profile found by ERIC-PCR and the IS-PCR profile, with the exception of 2 strains isolated from slaughterhouses. The 2 dissimilar Hadar isolates were associated with turkeys slaughtered in the same slaughterhouse. Antibiotyping also gave a different reading in that human isolates were multiresistant and therefore differentiated, whereas all the other isolates shared a single resistance pattern.

For Heidelberg and Virchow, we identified at least one common pulsotype in avian and human isolates, which may indicate an avian source for human infection. Additionally, the SHIDXB0001 profile, identified in a human Heidelberg strain, had previously been found in the poultry chain.

Two different PFGE profiles were identified for the Virchow isolates. It is possible that isolates exhibiting a SVIRXB0005 profile may have spread from broiler chickens to consumers. This hypothesis is supported by our results where all strains isolated from slaughterhouses shared this profile. To date, 93 strains of this serovar have been recorded in the database and 24 different profiles have been identified.

4.2.2. Nonmatching Human and Avian Patterns. Although human illnesses due to Enteritidis, Infantis, Senftenberg, and Typhimurium are commonly linked to avian sources, we did not find any matching pulsotypes between the avian and human isolates of these serovars. This must be emphasized particularly for Enteritidis and Senftenberg, even though they tend to originate in laying hens rather than broilers [28, 31, 32]. However, Cardinale et al. [33] highlighted the genetic similarity of *S*. Enteritidis PFGE profiles from human and broiler sources in Senegal. We may add that the SENTXB0001 profile has already been encountered in isolates of human origin, as well as from poultry, pastries, cooked meals, sea products, and so forth.

5. Conclusion

Our study did not confirm an association between the main serotypes detected in humans and those recovered in poultry production. However, collectively, our results bring to light a

probable significant contribution of nontyphoidal *Salmonella* by avian species to human salmonellosis in the Constantine region. Since the majority of isolates belonged to serovars usually associated with poultry, and despite the very low number of isolates studied, we were able to confirm identical profiles among avian and human isolates. The development of a large monitoring programme is crucial for the surveillance of *Salmonella* in poultry and the improvement of public health in Algeria.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Occurrence, Persistence, and Virulence Potential of *Listeria ivanovii* in Foods and Food Processing Environments in the Republic of Ireland

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The aim of this study was to assess the occurrence of *L. ivanovii* in foods and food processing environments in Ireland, to track persistence, and to characterize the disease causing potential of the isolated strains. A total of 2,006 samples (432 food samples and 1,574 environmental swabs) were collected between March 2013 and March 2014 from 48 food business operators (FBOs) belonging to different production sectors (dairy, fish, meat, and fresh-cut vegetable). Six of the forty-eight FBOs had samples positive for *L. ivanovii* on at least one sampling occasion. *L. ivanovii* was present in fifteen samples (fourteen environmental samples and one food sample). All but one of those positive samples derived from the dairy sector, where *L. ivanovii* prevalence was 1.7%. Six distinguishable pulsotypes were obtained by PFGE analysis, with one pulsotype being persistent in the environment of a dairy food business. Sequence analysis of the *sigB* gene showed that fourteen isolates belonged to *L. ivanovii* subsp. *londoniensis*, while only one isolate was *L. ivanovii* subsp. *ivanovii*. Cell invasion assays demonstrated that the majority of *L. ivanovii* strains were comparable to *L. monocytogenes* EGDe in their ability to invade CACO-2 epithelial cells whilst four isolates had significantly higher invasion efficiencies.

1. Introduction

The genus *Listeria* is at present comprised of fifteen low G+C content Gram-positive species. These are the *Listeria* sensu stricto species *L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. ivanovii*, the distantly related species *L. grayi*, and the very recently described species *L. rocourtiae*, *L. fleischmannii*, *L. weihenstephanensis*, *L. floridensis* sp. nov., *L. aquatica* sp. nov., *L. cornellensis* sp. nov., *L. riparia* sp. nov., and *L. grandensis* sp. nov. [1, 2]. Of these, only *L. monocytogenes* and *L. ivanovii* are recognized as pathogenic for warm-blooded hosts. While *L. monocytogenes* causes a severe foodborne disease in humans as well as invasive infections in a range of other mammals, *L. ivanovii* is almost exclusively linked to infections in sheep and cattle, although sporadic cases of *L. ivanovii* associated human infections have been reported [3, 4].

Due to its foodborne transmission, research on *L. monocytogenes* has received special attention in the last decades. Indeed, studies on occurrence and distribution of *L. monocytogenes* in foods and food processing environments are numerous and report variable prevalence. As an example, recent surveys carried out in the United Kingdom [5], Greece [6], Sweden [7], Ireland [8, 9], and various countries in Europe (Austria, Romania, Spain, and the Slovak Republic) [10] have reported *L. monocytogenes* prevalence ranging from 2.5 to 38%. There is less information available in the literature on the occurrence and distribution of other *Listeria* species along the food chain, although it appears that, apart from *L. monocytogenes*, *L. innocua* is the most frequently isolated *Listeria* species [11, 12]. Regarding *L. ivanovii*, a few reports exist which describe a low occurrence, generally of <2% [11–13], although little or no information is available on its occurrence in Irish food industries.

Bacterial persistence, defined as repeated isolation of an indistinguishable (by pulsed field gel electrophoresis [PFGE]) isolate at sampling times greater than 6 months, is a great concern for food industries since it can lead to the repeated contamination of food with spoilage or pathogenic microorganisms and has been demonstrated to recurrently happen for strains of *L. monocytogenes* [14]. A similar phenomenon could also occur for other members of the genus *Listeria*, including *L. ivanovii*. In fact, a study by Vázquez-Villanueva et al. [15] has provided evidence for the persistence of a *L. ivanovii* subsp. *ivanovii* isolate in a Spanish cheese factory. These authors found a common PFGE pulsotype in both ewe's and goat's raw milk batches tested over a 6-month period and on the inner surfaces of raw milk bulk tanks and the milk dump tank at the cheese factory.

Both *L. monocytogenes* and *L. ivanovii* are facultative intracellular bacteria capable of crossing the intestinal barrier and proliferating within macrophages and epithelial and endothelial cells and ultimately inducing cell-to-cell spread [16]. Interestingly, it is well known that *L. monocytogenes* isolates vary considerably with respect to virulence capacity and disease causing potential, with some isolates being incapable of invading gastrointestinal cells due to the expression of a truncated virulence factor, internalin A [17, 18]. Whether similar heterogeneity in disease causing potential is also present in *L. ivanovii* remains unexplored.

The aim of this study was to assess the occurrence of *L. ivanovii* in foods and food processing environments in the Republic of Ireland, to track persistence of the isolates, and to characterize the disease causing potential of the isolated strains.

2. Materials and Methods

2.1. Detection of L. ivanovii in Food and Environmental Samples. From March 2013 to March 2014, a total of 48 food processing facilities from various food sectors, that is, dairy (18 facilities), meat (12 facilities), seafood (8 facilities), fresh-cut vegetable (6 facilities), and miscellaneous (4 facilities), were sampled bimonthly. The selection of food processing facilities allowed coverage of major geographic areas of the Republic of Ireland.

Sampling packs, which consisted of a polystyrene box (DS Smith, UK) containing six premoistened 3M sponge-stick swabs (Technopath, Ireland), a sterile liquid container (VWR, Ireland), two sterile bags (VWR, Ireland), two cable ties, and two ice packs, were sent to all participating food processing facilities. Food business operators (FBOs) received detailed instructions which included information on how to take swab samples, which areas to sample, the type of food samples required, and the packaging and shipment of the samples to the laboratory. For food samples, FBOs were instructed to send two food samples which were at the stage of being ready to be sent from the processing facility.

Every second month, FBOs took 6 environmental samples and sent them to the laboratory by overnight courier along with 2 food samples. Thirty-seven FBOs were initially enrolled in the monitoring programme and 11 further FBOs later showed their interest in joining the collaborative network at different stages during the sampling year. On the other hand, 3 FBOs no longer wished to take part in the analysis or went out of business and several other companies missed one or various sample submissions throughout the sampling period.

Samples were analyzed by following the ISO 11290-1 method for detection of *L. monocytogenes*, except that only one chromogenic agar was used. After the environmental swabs arrived at the laboratory, 100 mL of half Fraser broth (VWR, Ireland) was added to bags containing 3M stick-sponge swabs, after which they were incubated at 30°C for 24 h. Then, a 0.1 mL aliquot was transferred to 10 mL of full Fraser broth, which was further incubated at 37°C for 48 h. In addition, a 0.02 mL aliquot of the 1st enrichment broth was plated onto Agar Listeria according to Ottaviani and Agosti (ALOA) agar plates (Biomérieux, UK), which were incubated at 37°C for 48 h. After incubation of the full Frazer broth, 10 μL was streaked onto ALOA agar plates, which were again incubated at 37°C for 48 h. For liquid or food samples, 225 mL of half Fraser broth was added to 25 mL or 25 g of randomly selected analytical units of the food samples. Samples were then homogenized in a stomacher (Colworth Stomacher 400) for 4 min and incubated at 30°C for 24 h. Subsequently, analysis of samples was continued by following the same approach used for environmental samples. After incubation, ALOA agar plates were examined for typical *L. monocytogenes/L. ivanovii* colonies (blue-green colonies with opaque halo).

After confirmation of *L. monocytogenes/L. ivanovii* isolates (performed as described below) sampling results were regularly communicated to collaborating FBOs.

2.2. Molecular Characterization of L. ivanovii Isolates. Two characteristic *L. monocytogenes/L. ivanovii* colonies for each positive enrichment were streaked first onto Brilliance Listeria Agar (BLA) plates (Fannin, Ireland), which were incubated at 37°C for 48 h, and then onto Brain Heart Infusion (BHI) agar plates, which were incubated at 37°C for 24 h. Cryoinstant tubes (VWR, Ireland) were prepared by resuspending the bacterial mass from BHI agar plates and were kept at −20°C for bioconservation.

Isolates were differentiated as *L. monocytogenes* or *L. ivanovii* by multiplex PCR as described by Ryu et al. [19] and *L. ivanovii* were confirmed by *sigB* sequencing as described below. PFGE analyses with the restriction enzymes *Asc*I and *Apa*I were carried out on all confirmed *L. ivanovii* isolates according to the International Standard PulseNet protocol [20]. Isolate similarity dendrograms were generated for PFGE analysis using the BioNumerics version 5.10 software (Applied Maths, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimization settings of 1%, as previously described [21]. In addition, representative isolates from each pulsotype were subjected to real-time PCR analyses for differentiation of *L. monocytogenes* and *L. ivanovii* through amplification of *hly* as described by Rodríguez-Lázaro et al. [22] and of *actA* as described by Oravcová et al. [23]. The *sigB* gene of *L. ivanovii* isolates was amplified using Taq DNA polymerase

(Thermo Scientific, Ireland) with primers sigB-F (AATATATTAATGAAAAGCAGGTGGAG) and sigB-R (ATAAATTATTTGATTCAACTGCCTT) at 95°C for 2 min, followed by 30 cycles of 95°C for 1 min, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Ireland) and sequenced by Source Bioscience services. Phylogenetic relationships between sequences were analysed using the web service http://www.phylogeny.fr/ as described by Dereeper et al. [24].

2.3. Invasion of CACO-2 Cells by L. ivanovii Isolates. The epithelial cell invasion assay was based upon the protocol of Nightingale et al. [25]. CACO-2 human intestinal cells (originally derived from human colon adenocarcinoma) were routinely maintained and grown in Dulbecco Modified Eagle Medium (DMEM) (Sigma-Aldrich, Ireland), supplemented with 10% Foetal Bovine Serum (Gibco, Ireland), 1% Penicillin-Streptomycin (Sigma-Aldrich), and 1% nonessential amino acids (Sigma-Aldrich) in a 37°C incubator supplemented with 5% CO_2. Cells were counted using a haemocytometer and trypan blue exclusion to a cell density of 2×10^5 cells/mL of medium and seeded into each well of a 24-well tissue culture plate (Starstedt), in triplicate. Cells were allowed to grow to a confluency of 80% over 48 h. Twenty-four hours prior to the assay, cells were washed and incubated in antibiotic-free DMEM.

Cultures of *L. monocytogenes* EGDe, *L. monocytogenes* PMSC1, or *L. ivanovii* strains were grown overnight in BHI at 37°C with shaking. One mL of the overnight culture was subsequently pelleted by centrifugation and then washed in PBS, diluted to a final concentration of 2×10^7 CFU/mL, and resuspended in antibiotic-free DMEM. Precise numbers of bacterial CFUs added to wells at T_0 were calculated subsequently following plate counts.

Growth medium was removed from the CACO-2 cells in each well and cells were washed once with sterile PBS and 1 mL of bacteria in antibiotic-free DMEM was added (giving a multiplicity of infection of 100). Cells were incubated for 1 h at 37°C/5% CO_2 to allow for internalisation of the bacteria. Subsequently, the bacterial inoculum was removed and the monolayer was washed once with sterile PBS. Fifty μg/mL gentamicin (Sigma) was resuspended in antibiotic-free DMEM, applied to the monolayer, and incubated for one further hour to kill extracellular bacteria. This was followed by lysis of the entire monolayer with ice cold sterile water containing 0.1% of TritonX-100. One hundred μL of the lysate was serially diluted and plated onto BHI agar (in triplicate for each well) which was incubated at 37°C overnight.

Data were expressed as mean ± SEM of at least three biological replicate samples. Data were transformed to log base ten prior to one-way Analysis of Variance (ANOVA) which was used to test the significance of differences in three or more groups followed by a post hoc test (in this case, Dunnett). In all cases, $P < 0.05$ was considered to be statistically significant. Graphs and statistical calculations were prepared using GraphPad Prism 5 (San Diego, California).

3. Results

From March 2013 to March 2014 a total of 2,006 samples (1,574 environmental samples and 432 food samples) were analyzed following the ISO 11290-1 standard methodology. *L. ivanovii* was present in fifteen of the 2,006 samples tested (prevalence of 0.75%), accounting for 14 environmental samples and one food sample. All but one positive environmental sample derived from processing facilities of the dairy sector, where *L. ivanovii* prevalence was 1.7%. These isolates were obtained from nonfood contact surfaces such as drains, floors, and pooled water on floors. The nondairy isolate was obtained from a seafood processing environment (floor), while the positive food sample was obtained from meat sausages. No positive samples were observed in processing facilities of the fresh-cut vegetable sector. It is important to note that only six of the forty-eight processing facilities analyzed had samples positive for *L. ivanovii* on at least one sampling occasion, with prevalence rates at those six facilities ranging from 1.8% to 13.1% (Table 1).

PFGE analysis was performed for all confirmed *L. ivanovii* isolates in order to track persistence events in the food processing environment (Figure 1). Six distinguishable pulsotypes were observed. In two dairy processing facilities (FBO 1 and FBO 12), *L. ivanovii* strains with indistinguishable PFGE profiles were isolated at various sampling times during the monitoring programme. For FBO 1, *L. ivanovii* isolates belonging to the same pulsotype were obtained from drains, floors, and pooled water on floors in May 2013, September 2013, November 2013, January 2014, and March 2014 (10-month persistence). For FBO 12, two *L. ivanovii* strains with indistinguishable PFGE profiles were isolated from drains in March 2013 and July 2013.

In order to characterize the *L. ivanovii* isolates at the subsp. level, the *sigB* gene was sequenced for representatives of the six distinguishable pulsotypes (Figure 2). Analysis of *sigB* sequences showed that five of the six pulsotypes (which correspond to 14 of the 15 positive samples) belonged to *L. ivanovii* subsp. *londoniensis*, while the remaining pulsotype (T6, with only one strain isolated from meat sausages) was *L. ivanovii* subsp. *ivanovii*.

When incorporating the PFGE profiles obtained in the current study to the *Listeria* spp. collection of profiles available at Teagasc Food Research Centre Moorepark, it became apparent that several isolates originally confirmed as *L. monocytogenes* by following the real-time PCR approach described by Rodríguez-Lázaro et al. [22] presented PFGE profiles indistinguishable from the ones obtained in this study. Some of these strains were analyzed by multiplex PCR and actually confirmed as *L. ivanovii* (data not shown). Subsequently, the real-time PCR protocol described by Rodríguez-Lázaro and coauthors was applied to representative strains of the six pulsotypes observed in the present study (Figure 3(a)). Amplification of the target *hly* gene occurred for both *L. monocytogenes* positive control strains used, with Ct values of 17.9 and 18.2, while late amplification of the target gene was observed for the *L. ivanovii* isolates tested, with Ct values ranging from 26.1 to 32.7. In addition, the real-time PCR meth odology described by Oravcová et al. [23] for confirmation

Table 1: Occurrence and pulsed field gel electrophoresis characterisation of isolates from *L. ivanovii* positive samples, listed according to processing facility, sampling month, sample type, and pulsotype, for example, T1. Empty white boxes indicate no *L. ivanovii* detected in submitted samples. "—" indicates nonsubmission of samples during that sampling month.

Facility number	% positives	March 13		May 13		July 13		September 13		November 13		January 14		March 14	
		Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods
							Dairy								
1	13.1			**T3**[*]				**T3**		**T3**		**T3**		**T3**	
2	0														
3	0														
4	0														
5	0														
6	2.4							T5				—	—		
7	0	—	—							—	—				
8	0														
9	0														
10	4					T2								T4	
11	0	—	—			—	—	—	—			—	—		
12	4.5	T1				T1		—	—			—	—		
13	0														
14	0														
15	0									—	—	—	—	—	—
16	0														
17	0	—	—	—	—	—	—					—	—		
18	0	—	—	—	—	—	—							—	—
							Meat								
19	0	—	—					—	—	—	—	—	—	—	—
20	0														
21	0														
22	1.8										T6				
23	0	—	—	—	—	—	—								
24	0	—	—	—	—	—	—								
25	0	—	—	—	—	—	—			—	—	—	—		
26	0			—	—	—	—	—	—	—	—	—	—	—	—
27	0									—	—	—	—		
28	0									—	—				
29	0			—	—					—	—	—	—		
30	0									—	—				
							Seafood								
31	0							—	—						
32	0							—	—						
33	0					—	—	—	—						
34	0														
35	0													—	—
36	0														
37	12.5	—	—	—	—	—	—	—	—	T4		—	—	—	—
38	0	—	—	—	—	—	—								

Table 1: Continued.

Facility number	% positives	March 13		May 13		July 13		September 13		November 13		January 14		March 14	
		Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods	Environment	Foods
Vegetable															
39	0														
40	0							—	—	—	—	—	—	—	—
41	0														
42	0					—	—								
43	0														
44	0											—	—		
Miscellaneous															
45	0														
46	0							—	—	—	—	—	—		
47	0	—	—	—	—	—	—								
48	0									—	—				

*Pulsotypes which persist in a single facility (isolated at least 6 months apart) are in bold.

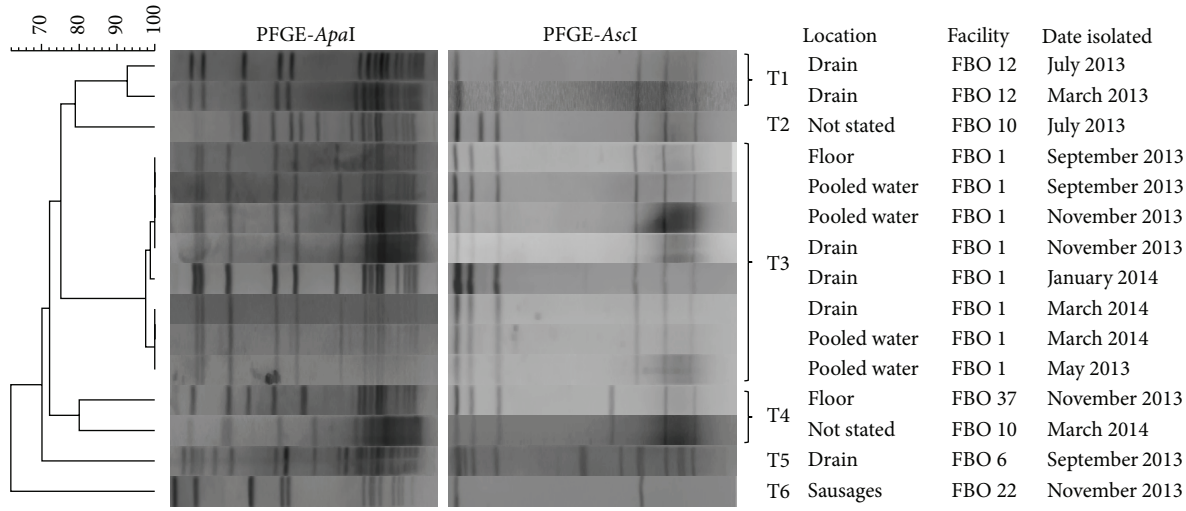

Figure 1: Dendrogram of PFGE pulsotypes of *Listeria ivanovii* isolates obtained from food and processing environment samples from the Republic of Ireland analyzed from March 2013 to March 2014.

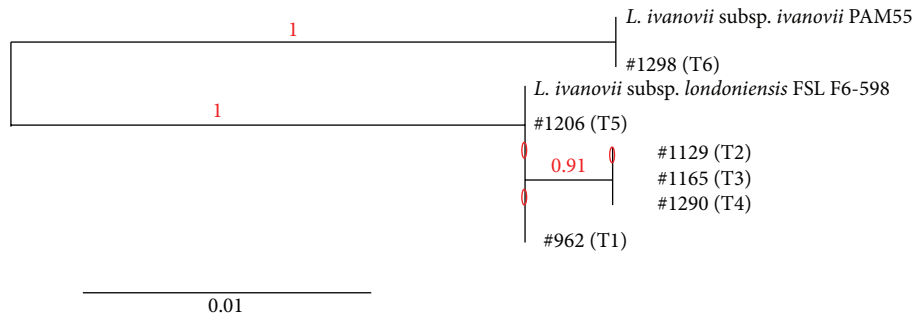

Figure 2: Phylogenetic tree (based on the sequence of the *sigB* gene) for the reference *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* strains and representatives of the six *L. ivanovii* pulsotypes found in the current study.

of *L. monocytogenes* based on the amplification of the *actA* gene was also tested with representative strains of the six *L. ivanovii* pulsotypes, and similarly late amplification events occurred, with Ct values ranging from 26.8 to 35.32, in contrast to Ct values of 18.4 and 20.0 observed for *L. monocytogenes* isolates tested (Figure 3(b)).

In order to determine the ability of various *L. ivanovii* strains to invade gastrointestinal epithelial cells, a standardized CACO-2 invasion assay [25] was carried out. Representative strains from 4 of the 6 pulsotypes were compared to an invasive laboratory strain of *L. monocytogenes* (strain EGDe) as well as a noninvasive *L. monocytogenes* strain carrying a defined premature stop codon in the *inlA* gene (PMSC1) [25]. The assay clearly differentiates between invasive and noninvasive *L. monocytogenes* isolates (Figure 4) and invasion efficiency of wild-type *L. monocytogenes* and the PMSC1 strain were roughly equivalent to results in previous studies [25, 26]. *L. ivanovii* strains were generally highly invasive with 7 out of 9 strains demonstrating levels of invasion that were equal to or higher than those of *L. monocytogenes* EGDe. Two strains (1261 and 1167) were moderately less invasive than *L. monocytogenes* EGDe, but none of the isolates demonstrated an invasion phenotype that was comparable to the PMSC1 *L. monocytogenes* isolate. Interestingly, four *L. ivanovii* isolates (1017, 1165, 1262, and 1290) were significantly ($P < 0.05$) more invasive than *L. monocytogenes* EGDe.

4. Discussion

The occurrence of *L. ivanovii* in foods and food processing environments was evaluated for the first time in the Republic of Ireland by bimonthly testing, over a one-year period, samples from forty-eight processing facilities. The observed *L. ivanovii* prevalence was in general low (0.75%). This agrees with the few reports available in the literature which also describe low *L. ivanovii* prevalence in the range 0–2% [11–13]. However, the results showed that *L. ivanovii* occurrence depended on the food sector. Thus, while a higher prevalence of 1.7% was observed for the dairy sector, very low prevalences (0.2% and 0.3%, resp.) were found for the meat and seafood sectors and no positive samples at all were obtained for the fresh-cut vegetable industry sector (278 samples analysed). It is important to note that *L. ivanovii* predominantly infects small ruminants and cattle, which can act as reservoirs. Ruminants can carry *L. ivanovii* and

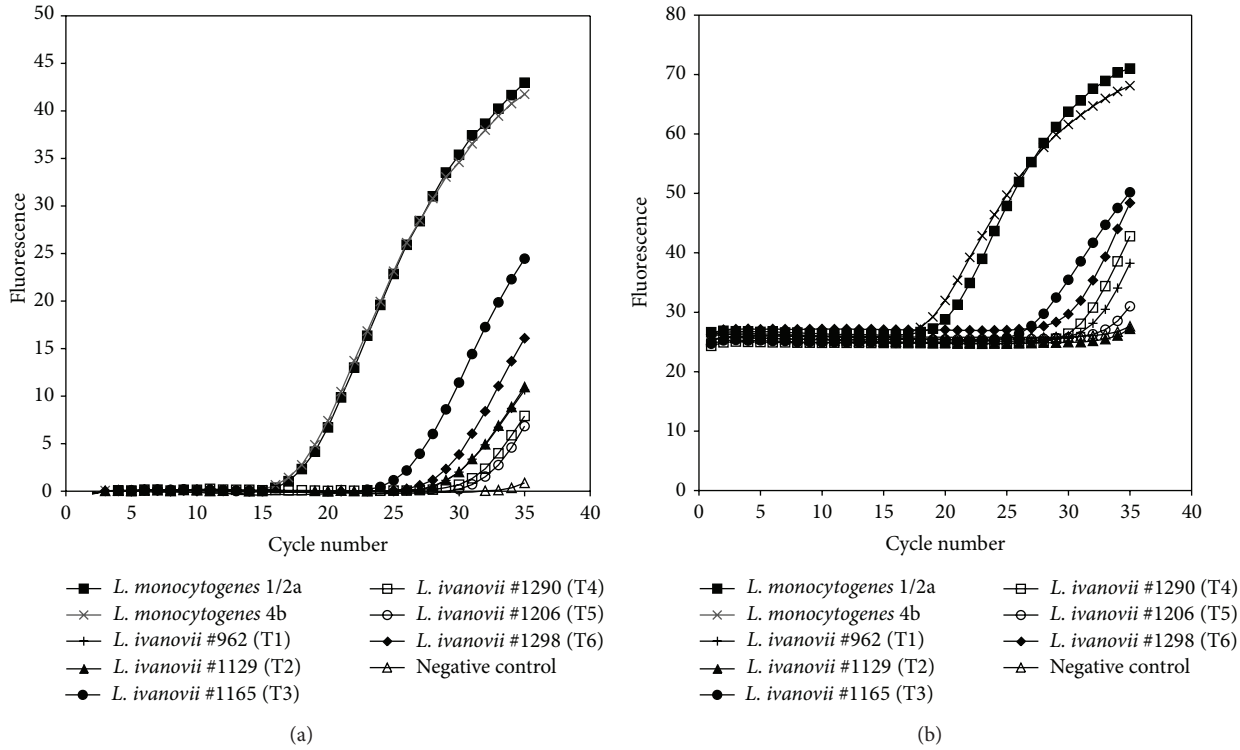

Figure 3: Amplification plot for *hly* (a) and *actA* (b) in *L. ivanovii* following the rt-PCR methodology described by Rodríguez-Lázaro et al. [22] and Oravcová et al. [23], respectively.

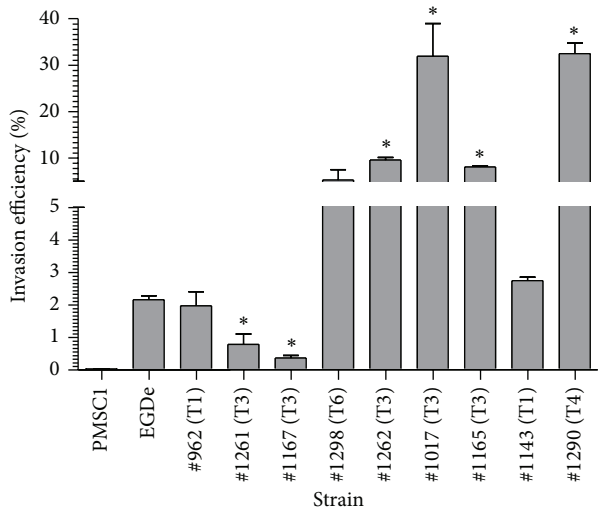

Figure 4: Invasive potential of wild-type *L. ivanovii* isolates in a CACO-2 epithelial cell assay. The strains were incubated with CACO-2 cells *in vitro* for one hour and levels of bacterial invasion were subsequently measured. For comparison, invasive (EGDe) and noninvasive (PMSC1) strains of *L. monocytogenes* were also examined. Data represents % invasion efficacy (relative to *Listeria* numbers initially added per well). Statistical significance was determined using one-way ANOVA and the Dunnett post hoc test with all strains compared to *L. monocytogenes* EGDe ($^*P < 0.05$). All strains displayed statistically higher ($P < 0.05$) levels of invasion efficiency relative to the PMSC1 strain.

contamination of milk can occur. Interestingly, three of the four dairy business operators that had positive samples (FBO 1, FBO 10, and FBO 12) produce cheese using milk from their own herds of cows or goats. Farming activity is carried out in those cases at facilities close to the cheese making facilities. This may potentially pose a further risk of processing environment contamination by *L. ivanovii*.

A survey regarding *L. monocytogenes* occurrence was conducted in parallel and showed that *L. monocytogenes* was present in 4.6% of samples analysed, with similar rates in food and environmental samples [9]. In most sampling occasions when *L. ivanovii* was detected no *L. monocytogenes* contamination was observed. However, there were three sampling occasions (Facility number 1: Environment, May 13; Facility number 1: Environment, November 13; Facility number 22: Foods, November 13) at which both *L. ivanovii* and *L. monocytogenes* isolates were identified, and in the particular case of Facility no. 1 both *L. ivanovii* and *L. monocytogenes* were isolated from the same samples (a drain and pooled water in the wash room) on November 13.

Molecular analysis of *L. ivanovii* isolates obtained throughout the monitoring programme showed that fourteen of the fifteen isolates (including all dairy isolates) belonged to *L. ivanovii* subsp. *londoniensis*, while only an isolate from meat sausages was *L. ivanovii* subsp. *ivanovii*. Interestingly, all environmental isolates were *L. ivanovii* subsp. *londoniensis*, while the only food isolate was *L. ivanovii* subsp. *ivanovii*. Whether *L. ivanovii* subsp. *londoniensis* is widely more

prevalent in the environments than *L. ivanovii* subsp. *ivanovii* or this is a particular phenomenon observed in processing facilities in Ireland remains to be elucidated.

Persistence of *L. ivanovii*, considered for this study as the detection of isolates with indistinguishable PFGE profiles at times six months or more apart, was observed for a cheese processing facility (FBO 1), where a persistent *L. ivanovii* subsp. *londoniensis* pulsotype (T3) was detected repeatedly over a 10-month period (from May 2013 to March 2014) in several nonfood contact environments (drains, floors, and pooled water on floors). In addition, another pulsotype (T1), which cannot yet be considered as persistent, was found in drains of a cheese factory (FBO 12) at times four months apart (March to July 2013). These two cheese processing facilities were the ones with the highest *L. ivanovii* occurrence (13.1% and 4.5%, resp.). Long-term survival of strains in a food processing facility, such as these, confers a higher risk of bacterial transfer to food and therefore a higher risk of human exposure to the microorganism. Bacterial persistence in food processing environments can be due to the existence of harborage sites that are colonized by bacteria and cannot be effectively cleaned or disinfected or can be due to the enhanced ability of some particular strains to grow or survive and therefore persist in industrial settings [27]. Thus, strains with increased resistance to sanitizers, higher adaptability to stress, or better ability to form biofilms might be better suited to persist in inhospitable environments such as those prevailing in food industries. Persistence of *L. ivanovii* in food processing environments has been also previously reported by Vázquez-Villanueva et al. [15] who identified a persistent *L. ivanovii* subsp. *ivanovii* pulsotype from ewe's and goat's raw milk samples from asymptomatic animals at farm level and from swabs obtained from the inner surfaces of raw milk truck tanks and the milk dump tank at the cheese factory level.

The current study also gives evidences that misidentification of *L. ivanovii* isolates as *L. monocytogenes* could occur when following the standard methodology for detection of *L. monocytogenes* in food and environmental samples. *L. ivanovii* strains are phosphatidylinositol-specific phospholipase C positive, and as such they grow in standard selective *L. monocytogenes* chromogenic agar plates forming colonies with the same characteristics as *L. monocytogenes* (blue-green colonies surrounded by an opaque halo on ALOA plates). Genes within the *prfA* virulence gene cluster are habitually used as target genes for *L. monocytogenes* confirmation PCR methodologies (e.g., *hly* and *actA*). The *prfA* virulence gene cluster is present between the *prs* and *ldh* genes in the pathogenic *L. monocytogenes* and *L. ivanovii* but is absent from the nonpathogenic *Listeria* species [28]. Two widely used rt-PCR methodologies specifically designed for the detection and quantification of *L. monocytogenes* and based on the amplification of the *hly* and *actA* genes [22, 23] were applied to the set of *L. ivanovii* strains isolated in the present study. The results showed that a late amplification (but earlier than the negative control) of both target genes occurred for *L. ivanovii* isolates, which could lead to an erroneous interpretation of results. Indeed, the Teagasc Food Research Centre Moorepark culture collection contained various strains originally classified as *L. monocytogenes* by following the approach described by Rodríguez-Lázaro et al. [22] that were subsequently identified as *L. ivanovii* during the course of this study. These results show the need for fine-tuning of the currently available molecular methodologies for confirmation of *L. monocytogenes*. Incorporation of such molecular tools able to rapidly and successfully discriminate *L. ivanovii* from *L. monocytogenes* is also advisable when implementing monitoring programmes focused on *L. monocytogenes*.

L. ivanovii is known to cause disease predominately in ruminants but has been associated on occasions with human disease [3, 4] and is considered to be a potential opportunistic pathogen of humans [4]. To date, studies examining the virulence characteristics of *L. ivanovii* have examined individual reference strains rather than collections of isolates. These studies indicate that *L. ivanovii* is capable of cellular invasion, often at levels in excess of *L. monocytogenes* [29–31]. *L. ivanovii* is also capable of lysis of the host cell phagosome and actin polymerization but is perhaps less effective than *L. monocytogenes* in cell-to-cell spread and intracellular multiplication [29, 31, 32]. The findings of this study support previous studies and demonstrated that some wild-type isolates of *L. ivanovii* are more invasive than a clinical *L. monocytogenes* reference isolate (EGDe). Indeed, the majority of isolates in this study were capable of highly effective cellular invasion, suggestive of some degree of disease causing potential. Further analysis is needed to ascertain the precise disease risk associated with these strains but the results suggest that such isolates may pose a health risk for immunocompromised individuals [4].

In conclusion, *L. ivanovii* prevalence in foods and food processing environments in the Republic of Ireland is low but cannot be considered negligible in processing facilities from the dairy sector, where contamination of environments through contaminated raw milk and persistence of isolates with good abilities to grow/survive in industrial settings in particular environments can occur, leading to a higher risk of contamination of processed foods. Although *L. ivanovii* is mainly linked to infections in sheep and cattle, recent reports have highlighted its disease causing potential in humans [3, 4] and the findings of this study demonstrated that the strains described are capable of invasion of human epithelial cells *in vitro*. These findings emphasize the need for dairy processors to be vigilant in order to avoid potential public health risks associated to *L. ivanovii* contamination.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Population Diversity of *Campylobacter jejuni* in Poultry and Its Dynamic of Contamination in Chicken Meat

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This study aimed to analyse the diversity of the *Campylobacter jejuni* population in broilers and to evaluate the major source of contamination in poultry meat. Eight rearing cycles over one year provided samples from three different broiler farms processed at the same slaughterhouse. A total of 707 *C. jejuni* were isolated from cloacal swabs before slaughter and from the breast skin of carcasses after slaughter and after chilling. All suspected *Campylobacter* colonies were identified with PCR assays and *C. jejuni* was genotyped by sequence analysis of the *flaA* short variable region (SVR) and by pulsed-field gel electrophoresis (PFGE) using *SmaI* enzyme. Phenotypic antibiotic resistance profiles were also assayed using minimal inhibitory concentration (MIC). The flocks carried many major *C. jejuni* clones possibly carrying over the rearing cycles, but cross contamination between farms may happen. Many isolates were resistant to fluoroquinolones, raising an issue of high public concern. Specific *Campylobacter* populations could be harboured within each poultry farm, with the ability to contaminate chickens during each new cycle. Thus, although biosecurity measures are applied, with a persistent source of contamination, they cannot be efficient. The role of the environment needs further investigation to better address strategies to control *Campylobacter*.

1. Introduction

Campylobacter is the most common cause of bacterial gastroenteritis in Europe. The incidence of human campylobacteriosis is increasing worldwide, as well as the number of isolates resistant to fluoroquinolones which are one of the primary classes of antimicrobials used to treat *Campylobacter* infection in human therapy and thus considered of high public concern [1]. In the European Union, *Campylobacter* is still the most commonly reported cause of bacterial foodborne illness with a notification rate of 55.49 cases per 100,000 of population in 2012 [2]. Poultry is a natural reservoir of *Campylobacter* species, constituting the most important source of human infection. The consumption of undercooked poultry meat or the mishandling of raw poultry products is considered to be the main risk factors associated with human campylobacteriosis [3–5].

The prevalence of *Campylobacter* in broiler chicken flocks ranges from 3 to 90% depending on their location [6, 7] and the isolation rates within positive flocks at slaughter are high (around 80%) [8–10]. Recent studies have reported that the prevalence of *Campylobacter* in retail chicken products ranges from 90 to 100% across several countries [11, 12]. *Campylobacter* colonization in chickens takes place at poultry farms, approximately 7 days after hatching [13], while widespread carcass contamination occurs at the slaughterhouse, especially from cross contamination by intestinal contents after the evisceration phase or from dirty surfaces [14]. Nevertheless, there have been few studies on the contamination of poultry carcasses from the farm through the entire production chain up to the retailer [15, 16] so the contamination routes in broiler flocks are still unknown.

The objective of the present study was to perform a comprehensive molecular characterization of *C. jejuni* isolated from poultry on the farm and during the slaughter process. Different typing methods, such as PFGE and *flaA*-SVR sequencing, will be used to trace the contamination of

chicken products and to investigate the potential of specific isolates to persist or be predominant in the poultry production. PFGE has been successfully applied to track *Campylobacter* during poultry production [16–18] and, together with *flaA*-SVR sequencing, it represents a highly discriminatory method for a better understanding of *Campylobacter* population structures. In addition, antibiotic susceptibility will also be investigated to determine the resistance pattern of *Campylobacter* that spread from chickens to humans along the poultry food chain, although the correlation between resistant bacteria in people and the use of antibiotics in feed is still a matter of debate [19].

2. Materials and Methods

2.1. Broiler Farms. Three different broiler farms (A, B, and C), randomly selected in the Abruzzo region of central Italy and spaced about 40 kilometres apart in a narrow zone, were enrolled in the study. The farms were managed similarly as part of the same integrated broiler company under good hygiene practices, rearing flocks of 40,000–60,000 birds with an average age at slaughter of 38–42 days.

2.2. Experimental Set-Up. Four different flocks were monitored on farm A, and two flocks each on farms B and C, amounting to a total of eight different rearing cycles under study between July 2011 and July 2012 with detailed dates shown in Table 1. For each flock, one day before slaughter, 50 different chickens, individually identified by leg rings, were randomly chosen and cloacal swabs taken (F), which were transported immediately to the laboratory using Ames transportation medium. The following day, the birds were transported 50 kilometres to the company abattoir, where samples were taken after slaughter (S) and after the chilling process (C). Samples S and C consisted of breast skin sampled under aseptic conditions, which were transported to the laboratory in a portable cooler at 2–4°C for immediate processing. The flocks tested were the first to be slaughtered on these days, using a slaughter line disinfected after the last batch processed on the previous day.

2.3. Culture Conditions and PCR Assays. A total of 1,720 samples were processed during the whole project and *Campylobacter* was recovered from carcass samples after the enrichment and the enumeration phases, according to parts 1 and 2, respectively, of the NF EN ISO 10272 standard procedure [20, 21]. The isolates were cultured on Columbia blood agar and incubated at 42°C for 48 h in a microaerophilic atmosphere. After a preliminary phenotypic characterization, suspected colonies were confirmed as thermotolerant *Campylobacter* and identified to species level using a multiplex PCR, as described previously by Di Giannatale et al. [22]. Genomic DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Isolates were stored in a Microbank (Pro-Lab Diagnostics Canada, Richmond Hill, ON, Canada) at 80°C until further analysis.

2.4. PFGE. Pulsed-field gel electrophoresiswas performed according to the 2009 U.S. PulseNet protocol for *Campylobacter* [23]. Briefly, bacteria previously identified as *Campylobacter* by PCR were subcultured on Columbia blood agar at 42°C for 2 days in a microaerophilic atmosphere and embedded in agarose blocks (Seakem Gold Agarose, Lonza, Rockland, ME, USA). The blocks were then lysed, washed, digested with 25 U of SmaI restriction endonuclease (Promega, Milan, Italy) at 25°C overnight, and subjected to pulsed-field electrophoresis in 1% agarose gel (Seakem Gold Agarose). PFGE was performed using a Chef Mapper XA (Bio-rad Laboratories, Hercules, CA, USA) and *Salmonella* serovar *Branderup* H9812 was used as standard molecular weight size. After electrophoresis, the gel was stained with Sybr Safe DNA gel stain (Invitrogen, Waltham, MA, USA) and photographed with a transilluminator (Alpha Innotech, San Leandro, CA, USA). For image analysis, Bionumerics v. 6.6 software (Applied Maths, Sint Martens Latem, Belgium) was used to identify the clusters of closely related or identical patterns. Pair comparisons and cluster analyses were carried out using the Dice correlation coefficient (position tolerance, 1.0%) and the unweighted pair group mathematical average (UPGMA) clustering algorithm. PFGE clusters were arbitrarily defined at a similarity level of 60% [24]. Untypeable isolates were not included in the analysis.

2.5. flaA SVR Sequencing. Typing was performed by amplifying the *flaA*-SVR using primers as described by Nachamkin et al. [25], followed by sequencing of the PCR product. Amplification products were verified by gel electrophoresis. PCR products were purified by using ExoSAP-IT reagent (GE Healthcare, Santa Clara, CA, USA) and sequenced using the BigDye Terminator v.3.1 Cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. After sequencing, DNA was purified with ethanol precipitation using the Agencourt CleanSEQ kit (Beckman Coulter, Brea, CA, USA). Sequencing products were analysed with a Genetic Analyzer 3500 (Life Technologies, Paisley, UK). The nucleotide sequences were compared with the *C. jejuni flaA* database (http://pubmlst.org/campylobacter/) and allele numbers were assigned accordingly. Confirmed sequences were aligned using MEGA 4 software [26]. For new *flaA*-SVR alleles, DNA trace files were submitted to the database administrator for confirmation. The peptide sequences were translated from the DNA sequences and named according to the Oxford database available at http://pubmlst.org/campylobacter/ The genetic diversity and the com-parison between the molecular methods were determined using the Simpson's diversity index (SDI) and the adjusted Rand index (aRI) via the online tool available at the Comparing Partitions website (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Home).

2.6. Antimicrobial Susceptibility. Campylobacter susceptibility to antibiotics was evaluated using the microdilution method by the "Sensititre" automated system (TREK Diagnostic Systems/Biomedical Service, Venice, Italy). Colonies were harvested in Columbia agar for 24 hours, inoculated

Table 1: Distribution of *flaA* and peptide types according to rearing cycle from farms A, B, and C.

	Peptide type	*flaA type*	12/12/2011	12/02/2012	10/05/2012	17/07/2012	Proportion	Number of strains
Farm A		1266	—	23 (24.46%)	—	—	9.23%	23
	1	260	1 (1.38%)	—	—	—	0.4%	1
		34	1 (1.38%)	—	—	—	0.4%	1
		49	—	—	1 (2.94%)	27 (55.10%)	11.24%	28
	368	1638	1 (1.38%)	67 (71.27%)	3 (8.82%)	19 (38.77%)	36.14%	90
	5	259	—	—	—	1 (2.04%)	0.4%	1
	8	117	—	—	—	1 (2.04%)	0.4%	1
		287	19 (26.38%)	—	17 (50.00%)	—	14.45%	36
		67	—	1 (1.03%)	4 (11.76%)	—	2%	5
	2	612	1 (1.38%)	—	—	—	0.4%	1
	10	1284	—	—	3 (8.82%)	—	1.21%	3
		1429	8 (11.11%)	—	—	—	3.21%	8
	103	327	1 (1.38%)	—	—	—	0.4%	1
	11	14	37 (51.38%)	1 (1.03%)	3 (8.82%)	—	16.46%	41
		17	3 (4.16%)	2 (2.12%)	2 (5.82%)	—	2.81%	7
		30			1 (2.94%)	1 (2.04%)	0.8%	2
	Total		**72**	**94**	**34**	**49**		**249**
			19/09/2011	17/11/2011	—	—		
Farm B	1	36	18 (15.65%)	106 (96.36%)	—	—	55.11%	124
		49	—	3 (2.72%)	—	—	1.33%	3
	11	11	1 (0.86%)	—	—	—	0.44%	1
	3	161	1 (0.86%)	—	—	—	0.44%	1
		5	—	1 (0.90%)	—	—	0.44%	1
	33	222	53 (46.08%)	—	—	—	23.55%	53
	8	287	42 (36.52%)	—	—	—	18.22%	42
	Total		**115**	**110**	**—**	**—**		**225**
			31/03/2012	12/06/2012	—	—		
Farm C	1	260	—	9 (10%)	—	—	4.1%	9
		23	—	1 (1.11%)	—	—	0.5%	1
		265	—	72 (80%)	—	—	32.9%	72
	8	117	3 (2.32%)	—	—	—	1.4%	3
		287	11 (8.52%)	2 (2.22%)	—	—	5.9%	13
	2	21	—	3 (3.33%)	—	—	1.4%	3
	33	222	1158 (89.14%)	—	—	—	52.5%	115
	9	239	—	3 (3.33%)	—	—	1.4%	3
		Total	**129**	**90**	**—**	**—**		**219**

in Mueller Hinton Broth supplemented with blood, and dispensed into Eucamp microtiter plates (TREK Diagnostic Systems/Biomedical Service), containing known scalar concentrations of the following antibiotics: gentamicin (0.12–16 μg/mL), streptomycin (1–16 μg/mL), ciprofloxacin (0.06–4 μg/mL), tetracycline (0.25–16 μg/mL), erythromycin (0.5–32 μg/mL), nalidixic acid (2–64 μg/mL), and chloramphenicol (2–32 μg/mL). The plates were then incubated at 42°C in a microaerophilic atmosphere for 24 hours. *C. jejuni* NCTC 11351 was included for the quality control in the MIC test.

3. Results

3.1. Campylobacter Prevalence. *Campylobacter* spp. was isolated in 1,081 of the samples. Further differentiation within the *Campylobacter* genus was obtained by PCR, resulting in 374 *C. coli* and 707 *C. jejuni*. The isolates were recovered from the different sources as follows: 281 *C. jejuni* and 56 *C. coli* from broiler flocks from the three farms, 366 *C. jejuni* and 248 *C. coli* from carcasses processed in the slaughterhouse, and 60 *C. jejuni* and 70 *C. coli* after chilling. At farm level, the prevalence of *C. jejuni* (65.77%) was significantly higher

($P < 0.05$, χ^2 test) than *C. coli* isolates (12.62%). All the flocks investigated from the different farms were positive for *Campylobacter* with high rates of prevalence, ranging from 58 to 90% of positive chicken (data not shown). In contrast, after chilling, the prevalence of *C. coli* (39.10%) was significantly higher ($P < 0.05$, χ^2 test) than *C. coli* groups at farm level (12.62%).

3.2. Typing. From farm A, 249 samples of *C. jejuni* were isolated during all four sampling periods (Table 1). Molecular investigation of the short variable region of the flagella revealed 16 different nucleotide types that corresponded to eight different peptide types (Table 1). Each flock was characterized by 5 to 9 different *flaA* types with one type predominant. In 6 instances, the same *flaA* type was recovered from different samples. The *flaA* type 1638 was isolated from all four rearing cycles, while *flaA* types 14 and 17 and *flaA* types 30, 49, 67, and 287 were isolated from 3 and 2 cycles, respectively (Table 1). From the analysis of isolates from the single flock of farm A, only 6 (4.38%) out of 137 *C. jejuni* isolated in the slaughterhouse did not belong to *flaA* types recovered from the farm. These remaining isolates showed the same fla type as those from the farm (Table 2). At a 60% similarity level, the PFGE clustering analysis revealed a high diversity within the isolates, grouping most of the isolates in three major clusters. The first cluster included 33 *C. jejuni* isolated from three rearing cycles (12.12.2011–10.05.2012–17.07.2012); the second cluster contained 130 *C. jejuni* isolated from all cycles analysed (12.12.2011–12.02.2012–10.05.2012–17.07.2012); the last cluster included 21 isolates from two cycles (12.12.2011–17.07.2012). All *C. jejuni* isolates in the three PFGE clusters were detected at farm, slaughter, and postchilling level. At a 100% similarity level, a dendrogram combining the data from farm A resulted in 56 different PFGE pulsotypes. Four pulsotypes comprised 49.57% (117/236) of the *C. jejuni* isolates from farm A, while 35 of the 56 pulsotypes included only a single *C. jejuni* isolate (Table 3). The polymorphisms resulting from the PFGE were higher than *flaA* typing with an SDI of 0.84 against 0.79; nevertheless the agreement between the methods resulted in an aRI of 0.44. A total of 225 strains of *C. jejuni* were isolated from farm B, 115 in the summer (19.09.2011) and 110 in the autumn (17.11.2011). Molecular investigation of the flagella determined seven different nucleotide types corresponding to five different peptide types (Table 1). Only *fla* type 36 was recovered in both rearing cycles analysed. All the isolates collected after chilling showed *fla* types previously detected in the live chicken. Six isolates out of 123 collected from the slaughterhouse featured four *fla* types (49, 11, 161, and 5) that were different from those collected on the farm (Table 2). At a 60% similarity level, the PFGE clustering showed a high variability with four major clusters. The first cluster included 80 *C. jejuni* isolated from two rearing cycles (19.09.2011–17.11.2011) obtained at farm, slaughter, and postchilling phases; the second cluster contained 96 *C. jejuni* from one flock (19.09.2011) but they were present in all the phases analysed; the third cluster included 26 isolates from one flock (17.11.2011) at farm and slaughter level; the last cluster included 16 isolates from two rearing cycles (19.09.2011–17.11.2011) obtained at the farm and slaughter phases. At a 100% similarity level, all the *C. jejuni* from farm B were clustered in 39 different pulsotypes with four that comprised 53.73% of the isolates and 22 pulsotypes represented by a single isolate (Table 3). The polymorphisms of PFGE showed an SDI of 0.927, higher than the *fla* typing value of 0.591, but agreed well with an aRI of 0.819. A total of 219 *C. jejuni* were recovered from farm C, 126 in the winter (31.03.2012) and 93 in the spring (12.06.2012). The *fla* SVR sequencing identified seven *fla* SVR sequences, corresponding to five peptide alleles (Table 1). Both samplings from this farm revealed the *fla* type 287 (Table 1). From the slaughterhouse, 4 out of 219 isolates provided two *fla* types not present in those *C. jejuni* from the cloacal swabs (Table 2). At a 60% similarity level, the PFGE clustering showed a high variability with three major clusters. The first cluster included 49 *C. jejuni* isolated from one rearing cycle (12.06.2012) collected during the farm, slaughter, and postchilling phases; the second cluster contained 11 *C. jejuni* isolated in two rearing cycles (31.03.2012–12.06.2012) during the farm and slaughter phases; the third cluster included 134 *C. jejuni* isolated in two rearing cycles (31.03.2012–12.06.2012) during the farm, slaughter, and postchilling phases. At a 100% similarity level, the samples from farm C were divided into 60 different pulsotypes, with three of them comprising 30.85% (58/188) of the isolates and 43 pulsotypes represented by a single isolate (Table 3). For PFGE, the SDI for this cycle was 0.944 while that for the *fla* type was 0.627 and the aRI displayed a fairly high value of 0.864. The distribution of *flaA* alleles and peptides isolates in the three farms A, B, and C is summarized in Figure 1. Sixty-nine isolates (9.76%) were untypeable with PFGE, appearing to be a case of DNA smearing rather than restriction.

3.3. Antimicrobial Susceptibility Tests. MIC and antimicrobial resistance of all *Campylobacter* isolates tested in this study are presented in Table 4. The MIC test revealed that 90% of the isolates were resistant to quinolones (NAL and CIP), but 98% were susceptible to chloramphenicol and streptomycin and 99% susceptible to gentamicin. Notably, 64% of the *Campylobacter* showed resistance to tetracycline, 18% showed resistance to erythromycin, and a few isolates were resistant to other antimicrobials such as chloramphenicol (1.2%), streptomycin (1%), and gentamicin (0.3%). Furthermore, resistance to erythromycin and tetracycline antimicrobials was significantly more frequent in *C. coli* compared with *C. jejuni* ($P < 0.05$, χ^2 test), whereas no differences were observed for the remaining antibiotics.

4. Discussion

Over the last five years, campylobacteriosis has become more prevalent in Europe. *Campylobacter* is found mostly in chicken meat with poultry and poultry farms playing a key role in the epidemiology of human infection [27, 28]. In Italy, a European survey showed a prevalence of *Campylobacter*-colonized broiler batches of 63.3% [9]. Similar prevalence levels in Italy (60%) have recently been obtained by other

Table 2: Number of *C. jejuni* grouped by *flaA* type, rearing cycle, and sampling point from farms A, B, and C.

(a)

	flaA type	12/12/2011			12/02/2012			10/05/2012			17/07/2012		
		F	S	C	F	S	C	F	S	C	F	S	C
Farm A	1266	—	—	—	19	4	—	—	—	—	—	—	—
	260	—	1	—	—	—	—	—	—	—	—	—	—
	34	—	1	—	—	—	—	—	—	—	—	—	—
	49	—	—	—	—	—	—	—	1	—	7	20	—
	1638	—	1	—	16	48	3	1	2	—	2	15	2
	259	—	—	—	—	—	—	—	—	—	—	1	—
	117	—	—	—	—	—	—	—	—	—	1	—	—
	287	9	10	—	—	—	—	5	11	1	—	—	—
	67	—	—	—	—	1	—	3	1	—	—	—	—
	612	—	1	—	—	—	—	—	—	—	—	—	—
	1284	—	—	—	—	—	—	2	1	—	—	—	—
	1429	2	6	—	—	—	—	—	—	—	—	—	—
	327	—	1	—	—	—	—	—	—	—	—	—	—
	14	15	22	—	—	1	—	1	2	—	—	—	—
	17	2	1	—	—	1	1	2	—	—	—	—	—
	30	—	—	—	—	—	—	—	—	1	—	1	—
	Total	**28**	**44**	**0**	**35**	**55**	**4**	**14**	**18**	**2**	**10**	**37**	**2**

(b)

	flaA type	19/09/2011			17/11/2011		
		F	S	C	F	S	C
Farm B	36	3	13	2	45	49	12
	49	—	—	—	—	3	—
	11	—	1	—	—	—	—
	161	—	1	—	—	—	—
	5	—	—	—	—	1	—
	222	21	29	3	—	—	—
	287	6	26	10	—	—	—
	Total	**30**	**70**	**15**	**45**	**53**	**12**

(c)

	flaA type	31/03/2012			12/06/2012		
		F	S	C	F	S	C
Farm C	260	—	—	—	3	6	—
	23	—	—	—	—	1	—
	265	—	—	—	34	38	—
	117	—	3	—	—	—	—
	287	5	5	1	1	1	—
	21	—	—	—	—		3
	222	36	60	19	—	—	—
	239	—	—	—	2	1	—
	Total	**41**	**68**	**20**	**40**	**47**	**3**

F = cloacal swabs; S = slaughterhouse line; C = postchilling phase.

studies [29, 30]. The present study aimed to analyse the diversity of the *C. jejuni* population in poultry and to monitor the contamination process throughout the farm, slaughter, and postchilling phases. The results have shown a very diverse *C. jejuni* population, even though only three broiler farms from a narrow area were evaluated. A total of 25 *flaA*-SVR types and 11 *flaA* peptides were identified among the numerous isolates that were analysed, demonstrating the presence of a heterogeneous population. This is also supported by previous studies where isolates from different continents were

Table 3: Distribution of PFGE pulsotypesat 100% similarity according to sampling point from farms A, B, and C.

	PFGE pulsotypes	Cycles	Major PFGE pulsotype	F (Number of isolates/total number of samples)	S (Number of isolates/total number of samples)	C (Number of isolates/total number of samples)
Farm A	56	12.12.2011	A	(10/236)	(9/236)	(7/236)
			D	(7/236)	(5/236)	(1/236)
		22.02.2012	B	(22/236)	(31/236)	(5/236)
		17.07.2012	B	(5/236)	(1/236)	(1/236)
			C	(8/236)	(5/236)	(0/236)
Farm B	39	19.09.2011	E	(16/214)	(18/214)	(2/214)
			F	(5/214)	(12/214)	(8/214)
		17.11.2011	G	(10/214)	(14/214)	(0/214)
			H	(17/214)	(8/214)	(5/214)
Farm C	60	31.03.2012	I	(0/188)	(2/188)	(0/188)
			L	(14/188)	(5/188)	(0/188)
			M	(0/188)	(5/188)	(12/188)
		12.06.2012	I	(1/188)	(9/188)	(0/188)

Sampling point: F = cloacal swabs; S = slaughterhouse line; C = postchilling phase.

assessed by *flaA* SVR typing revealing a similar degree of diversity [31, 32]. Interestingly, we found a high individual prevalence of *Campylobacter,* in common with other studies [10]. Tracing back the *Campylobacter* for each flock showed that the major source of chicken meat contamination remains the flock itself. In the present study, only 10% of the isolates from the abattoir were distinguishable from the live chicken isolates, showing that there were few cases of contamination during slaughtering. Frequently, the most common *fla* types in live chickens were also the most common genotypes in the processed carcasses and this confirms results reported in previous studies [6, 10, 33, 34]. In a context where all flocks are contaminated, it seems that the slaughterhouse does not play an important role in carcass contamination. However, the situation completely changes when *Campylobacter*-free flocks meet contaminated flocks at the abattoir. It is therefore sound practice for contaminated poultry flocks to be slaughtered at the end of the working day to contain the cross contamination among the flocks. So diagnostic systems must be able to detect *Campylobacter* and distinguish uncontaminated from contaminated flocks. The potential of *Campylobacter* to carry over to succeeding rearing cycles would indirectly suggest its ability to survive within the broiler farm. A comparison of isolates from different samplings for farm A showed that seven *fla* alleles (83.94%) recurred over a period of almost 8 months. The overlaps of *fla* genotypes were minor for farms B and C, probably because of the short length of monitoring undertaken, although communities were also demonstrated by the carryover of alleles 36 and 287. To strengthen these findings, we also found that PFGE clustering at 60% of similarity grouped isolates from different rearing cycles. These isolates fell into the same PFGE cluster and featured the same *fla* allele suspected to be stable over time thus indicating that some isolates were successful in the broilers. The *fla* SVR analysis also showed that 38.57% of the isolates shared the same *fla* alleles among the three farms (Figure 1), although a limited correlation between the farms could be argued. *fla* allele 287, in particular, revealed a PFGE clustering, supporting the hypothesis that all the isolates were strongly related, independently of the farms (Figure 2). This could be explained by cross contamination, probably caused by objects that might transport *Campylobacter* within the broiler houses most likely during the thinning process. In the present study, the farms were managed as part of a vertically integrated supply chain. Generally, feed mills, breeding farms, hatcheries, and slaughterhouses are owned by the same company and it is probable that the same catching crew could cross contaminate the farms by using unclean crates. Monitoring these practices very thoroughly is required to better address these types of problem. Antibiotic resistance has been a long-standing problem in the field of human and veterinary medicine [31, 35–40] generally related to the indiscriminate use of antibiotics in prophylaxis and therapy or as a growth promoter [39]. Comparative studies of isolates from different geographical areas show a steady and alarming increase in resistance, even to the next generation molecules [31, 32, 37, 40–42]. Particularly worrying is the increase in the frequency of resistance against fluoroquinolones, particularly ciprofloxacin [10], which was confirmed in our study (90%). Moreover our results on resistance against nalidixic acid (90%) and tetracycline (64%) agreed with those in the EFSA Report [42] and other studies [31, 43], confirming this increasing trend. The susceptibility against chloramphenicol (1.2%), streptomycin (1%), and gentamicin (0.3%) could be probably attributable to the lack of extensive use of these drugs in Italy.

5. Conclusions

This study has revealed the usefulness of molecular methods for tracing *Campylobacter* contamination in the poultry supply chain. These data have provided more information on

Table 4: Antimicrobials, dilution ranges, and cut-off values used for MIC determination of *Campylobacter*.

Antimicrobials	MIC breakpoints (μg/mL)		Distribution % of MIC (μg/mL)											MIC_{50}	MIC_{90}	Number of resistant	Number of resistant	Number of resistant
	S	R	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	(μg/mL)	(μg/mL)	isolates (%)	*C. jejuni* (%)	*C. coli* (%)
Chloramphenicol	≤8	≥32						76.5	21	1.2	0	1.3		2	4	9 (1.2%)	8 (1.71%)	1 (0.34%)
Ciprofloxacin	≤1	≥4	7.2	0.4	0	0.1	0	1.9	90.4					4	4	696 (90.4%)	424 (90.79%)	272 (93.15%)
Erythromycin	≤0.5	≥8				72	8.5	1.2	0.4	0.3	0.5	17.1		0.5	32	139 (18%)	29 (6.20%)	110 (41.98%)*
Gentamicin	≤4	≥16		39	14.3	31	13.3	1.6	0.2	0.3	0.3			0.25	1	2 (0.3%)	2 (0.42%)	0
Nalidixic acid	≤16	≥32						6.6	0.7	0.7	1.6	8.4	82	64	64	697 (90%)	428 (91.64%)	269 (92.12%)
Streptomycin	≤2	≥8					66.5	18.2	14.3	0.4	0.6			1	4	8 (1%)	7 (0.14%)	1 (0.34%)
Tetracycline	≤4	≥16			33	1.3	0.2	0.5	0.2	0.8	64			16	16	492 (64%)	272 (58.24%)	220 (75.34%)**

S = sensible, R = resistant.

*Statistically significant versus *C. jejuni* group ($P < 0.05$, χ^2 test), **statistically significant versus *C. jejuni* group ($P \leq 0.01$, χ^2 test).

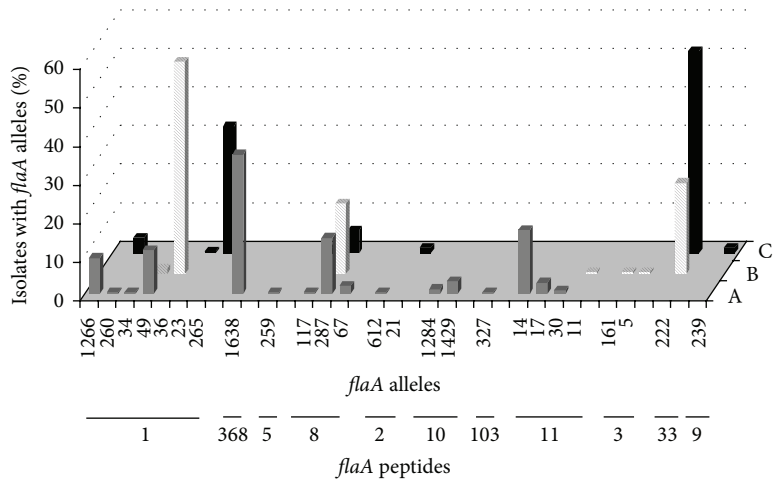
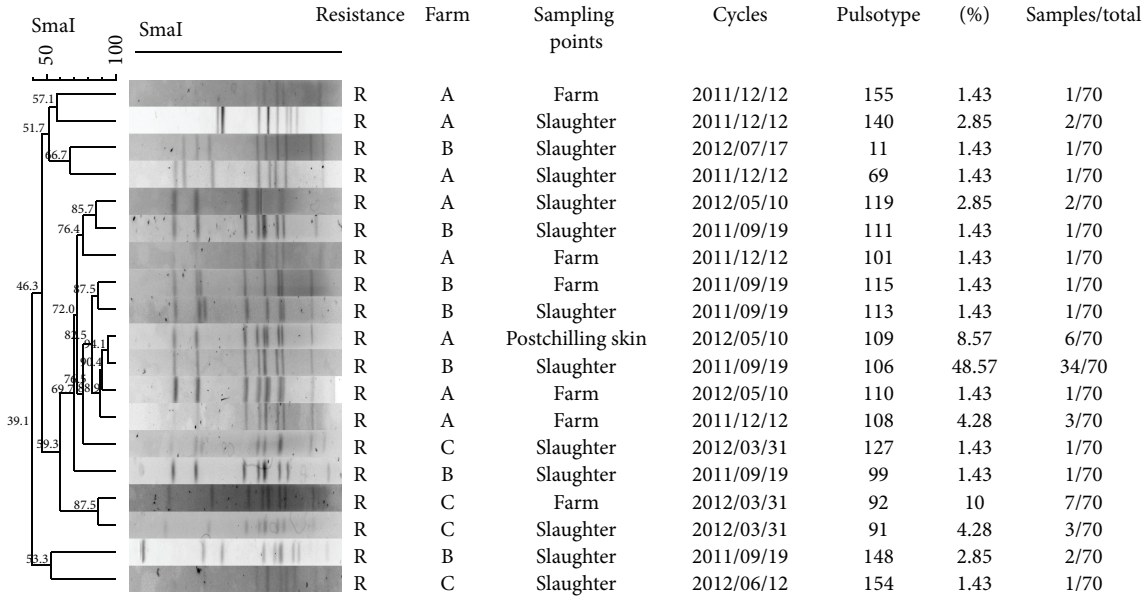


Figure 1: Distribution of *flaA* alleles and peptides (shown on the *x*-axis) from farms A, B, and C.



Resistance	Farm	Sampling points	Cycles	Pulsotype	(%)	Samples/total
R	A	Farm	2011/12/12	155	1.43	1/70
R	A	Slaughter	2011/12/12	140	2.85	2/70
R	B	Slaughter	2012/07/17	11	1.43	1/70
R	A	Slaughter	2011/12/12	69	1.43	1/70
R	A	Slaughter	2012/05/10	119	2.85	2/70
R	B	Slaughter	2011/09/19	111	1.43	1/70
R	A	Farm	2011/12/12	101	1.43	1/70
R	B	Farm	2011/09/19	115	1.43	1/70
R	B	Slaughter	2011/09/19	113	1.43	1/70
R	A	Postchilling skin	2012/05/10	109	8.57	6/70
R	B	Slaughter	2011/09/19	106	48.57	34/70
R	A	Farm	2012/05/10	110	1.43	1/70
R	A	Farm	2011/12/12	108	4.28	3/70
R	C	Slaughter	2012/03/31	127	1.43	1/70
R	B	Slaughter	2011/09/19	99	1.43	1/70
R	C	Farm	2012/03/31	92	10	7/70
R	C	Slaughter	2012/03/31	91	4.28	3/70
R	B	Slaughter	2011/09/19	148	2.85	2/70
R	C	Slaughter	2012/06/12	154	1.43	1/70

Figure 2: Dendrogram of *C. jejuni* SmaI PFGE patterns isolated in the three farms characterized by *flaA* allele 287 and antimicrobial resistance to fluoroquinolones.

the presence of *Campylobacter* clones that have adapted well to poultry and can survive on the farms. The question arises whether *Campylobacter* has an ecological niche that permits its survival. Several hypotheses have been debated but no data are available to evaluate water supplies and vectors such as flying insects or rodents as potential risk factors involved in the mechanism of contamination [6, 44]. Our results showed a highly diverse *C. jejuni* population in poultry, suggesting that its introduction or reintroduction on the farm may originate from different sources. Since the main source of poultry meat contamination was confirmed to be the flock, it is reasonable to suggest that *Campylobacter*-free meat could be achieved by reducing its prevalence at farm level.

Conflict of Interests

The authors declare no conflict of interests relating to this study.

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Research Article

Genetic Diversity and Incidence of Virulence-Associated Genes of *Arcobacter butzleri* and *Arcobacter cryaerophilus* Isolates from Pork, Beef, and Chicken Meat in Poland

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Incidence of 9 virulence-associated genes and genetic diversity was determined in 79 *A. butzleri* and 6 *A. cryaerophilus* isolates from pork, beef, and chicken meat. All *A. butzleri* isolates harboured the *tlyA* gene, and most of them carried *ciaB*, *mviN*, *pldA*, *cadF*, and *cj1349* genes. *ciaB* was found to occur with higher frequency in poultry if compared with pork ($p = 0.0007$), while *irgA* was more frequent in poultry than in beef ($p = 0.007$). All 6 *A. cryaerophilus* isolates harboured the *ciaB* gene, while *mviN* and *tlyA* were detected in 3 out of these isolates. Only one isolate carried the *cadF* gene. All beef-derived *A. cryaerophilus* isolates carried *ciaB*, *mviN*, and *tlyA* genes. *A. cryaerophilus* isolates from chicken meat harboured *ciaB* gene only. The pork-derived isolate harboured *ciaB* and *cadF* genes. Seventy-four genotypes were distinguished within 79 *A. butzleri* isolates. Nineteen from 21 isolates derived from beef and pork were found to be closely related to *A. butzleri* from chicken meat. Each of the 6 *A. cryaerophilus* isolates was found to have unique genotype. We demonstrated that closely related genotypes can spread within pork, beef, and chicken meat populations of *A. butzleri* but not *A. cryaerophilus*.

1. Introduction

Genus *Arcobacter,* formerly known as "aerotolerant *Campylobacter*," was included into the family Campylobacteraceae in 1991 [1] and currently consists of twenty species [2, 3]. Three *Arcobacter* species, that is, *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*, have been mainly associated with enteritis in humans and abortion in pigs [4–6]. *A. butzleri* has been classified as a serious hazard to human health by the International Commission on Microbiological Specifications for Foods in 2002 [7]. The bacteria have been isolated from food, mainly from products of animal origin, with the highest prevalence in poultry, followed by pork and beef [8–10]. Consumption of *Arcobacter*-contaminated food is regarded as the most likely source of human infection [11]. *Arcobacter* spp. were also isolated from drinking water reservoirs and sewage [12]. Recent evidence suggests that *Arcobacter* spp., especially *A. butzleri* and *A. cryaerophilus*, may be involved in human enteric diseases [4, 13, 14]. Infections involving *Arcobacter* spp. may result in abdominal pain with acute diarrhoea or prolonged watery diarrhoea [15].

Analysis of complete genome of *A. butzleri* ATCC (American Type Culture Collection) 49616 revealed some homologues to virulence genes identified in *Campylobacter* and other bacteria, including *E. coli* and *Vibrio* spp. Already identified virulence-associated genes of *A. butzleri* include *cadF, cj1349, ciaB, mviN, pldA, tlyA*, *irgA*, *hecA*, and *hecB* [16]. *cadF* and *cj1349* encode fibronectin-binding proteins; *ciaB* encodes *Campylobacter* invasive antigen (CiaB), which may contribute to host cell invasion; *hecA* encodes HecA protein,

a member of the filamentous hemagglutinin; *hecB* encodes a hemolysin activation protein; *tlyA* encodes hemolysin; *mviN* encodes essential protein MViN required for peptidoglycan biosynthesis; *pldA* encodes outer membrane phospholipase PldA associated with lysis of erythrocytes; and *irgA* encodes iron-regulated outer membrane protein IrgA. Although some authors reported on abundance of virulence factors, pathogenicity, and genetic diversity of food-derived *Arcobacter* isolates throughout the world, characteristics of *Arcobacter* spp. in Poland are still unknown [17–21].

The aim of this work was to determine the incidence of virulence-associated genes and genetic diversity of *A. butzleri* and *A. cryaerophilus* isolates from pork, beef, and chicken meat in Poland.

2. Materials and Methods

2.1. Bacterial Strains. Seventy-nine *A. butzleri* and 6 *A. cryaerophilus* cultures were isolated from meat samples, purchased from retail market in Lower Silesia region (Poland) as described previously [22]. Among 79 *A. butzleri* isolates, 58, 11, and 10 were obtained from chicken, beef, and pork samples, respectively. Within *A. cryaerophilus* cultures, 3 isolates were from beef, 2 were from chicken, and one was from pork meat. *Arcobacter* spp. isolates were cultured according to Houf et al. [23] with modifications, in *Arcobacter* broth (Oxoid) with selective supplement containing cefoperazone, amphotericin B, and teicoplanin (CAT, Oxoid). Additionally, novobiocin (32 mg/L), 5-fluorouracil (100 mg/L), and trimethoprim (64 mg/L) (Sigma) were added to the broth. After 48-hour incubation in aerobic atmosphere, at 30°C, the bacteria were subcultured on *Arcobacter* agar plates (supplemented with chemotherapeutics mentioned above) and in parallel on agar plates with defibrinated sheep blood (Oxoid). Phenotypically suspected colonies were transferred to blood agar plates and incubated in aerobic conditions for 48 h at 30°C. One *Arcobacter* spp. isolate per sample was taken for further characterization. The isolates were preserved by freezing in Cryobank (Mast Diagnostics) at −80°C.

2.2. Preparation of Bacterial DNA. Total DNA was isolated as described by Agersborg et al. [24]. Briefly, the bacteria from 1 mL overnight culture were pelleted by centrifugation and suspended in 200 μL of distilled water containing 1% Triton X-100. The mixture was boiled for 10 min, and then the tubes were centrifuged for 5 min at 13 000 ×g. The supernatant containing DNA was used in PCR.

2.3. Species Identification of Arcobacter Isolates. The isolates were identified at species level using multiplex PCR according to Houf et al. [25]. Amplification products were resolved in 1.5% agarose containing 0.5 μg/mL ethidium bromide and documented using GelDocXR System (BioRad, Hercules, CA). Each PCR run was performed using DNA from the reference CCM (Czech Collection of Microorganisms) 4826 *A. butzleri* and CCM 3933 *A. cryaerophilus* strains as positive controls and ATCC 33560 *Campylobacter jejuni* as a negative control.

2.4. Identification of Virulence-Associated Genes. *A. butzleri* and *A. cryaerophilus* isolates were PCR-screened for the presence of nine virulence-associated genes, such as *cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *irgA*, *hecA*, and *hecB*, as described by Douidah et al. [17]. The 10 μL aliquots of PCR products were resolved on 1.5% agarose gel and documented with a GelDoc XR documentation system (BioRad, Hercules, USA).

2.5. Genotyping of A. butzleri and A. cryaerophilus Isolates. A survey of the genetic diversity of the *A. butzleri* and *A. cryaerophilus* isolates was performed using ERIC PCR primers ERIC2 5′-AAGTAAGTGACTGGGGTGAGCG-3′ and ERIC1R 5′-ATGTAAGCTCCTGGGGATTCAC-3′ as described by Houf et al. [26]. The PCR was performed in a mixture containing 20 mmol/L Tris-HCl, pH 8.4, 3 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.2 mmol/L of each deoxyribonucleotide, 1.25 U of Taq DNA polymerase (Thermo Scientific, Poland), 50 pmole of each primer (Genomed, Warsaw, Poland), and 2 μL of DNA solution. Thirty-six PCR cycles were carried out according to the following scheme: denaturation at 94°C for 30 s, annealing at 35°C for 30 s, and elongation at 72°C for 120 s. The denaturation/annealing and annealing/elongation ramping rates were set to 5 minutes [27]. Amplicons were separated on 3% agarose gel at 170 V for 3 h. The gels were stained with ethidium bromide, visualized on a UV transilluminator, and photographed. The resulting profiles were analyzed using the software available on http://insilico.ehu.es/dice_upgma/ to generate dendrograms by UPGMA clustering, using Dice correlation.

2.6. Statistical Analysis. Mann-Whitney *U* test was applied to assess statistical significance of obtained data. Calculations were performed using Statistica 9.1 (StatSoft, Poland). p values below 0.05 were considered to be significant.

3. Results

3.1. Prevalence of Virulence-Associated Genes in A. butzleri and A. cryaerophilus Isolates. Screening of 79 *A. butzleri* isolates revealed that all of them harboured the *tlyA* gene. Most of the *A. butzleri* isolates carried *ciaB* (97%), *mviN* (97%), *pldA* (92%), *cadF* (89%), and *cj1349* (66%), while *hecB* (48%), *irgA* (46%), and *hecA* (30%) genes were less frequent (Table 1). *ciaB* was found to occur with higher frequency in poultry if compared with pork ($p = 0.0007$), while *irgA* was more frequent in poultry than in beef ($p = 0.007$).

Ten percent (8 out of 79) of the *A. butzleri* isolates harboured all nine genes, and most of them (7 isolates) derived from chicken meat. The carriage of 8 and 7 virulence-associated genes was reported in 44% (35 out of 79) of *A. butzleri* isolates. Most of them were found in chicken meat (28 isolates), followed by pork (4 isolates) and beef (3 isolates). Seventeen (22%), 14 (18%), and 2 (3%) *A. butzleri* isolates possessed 6, 5, and 4 virulence-associated genes, respectively (Figure 1). The isolates harbouring 3 (2 isolates) and 2 genes (1 isolate) were only reported in *A. butzleri* from beef and pork, respectively.

Table 1: Carriage of virulence genes by *A. butzleri* and *A. cryaerophilus* isolates.

Species	Origin/number of isolates	Percent of isolates harbouring virulence genes possessing specific gene								
		cadF	*cj1349*	*ciaB*	*irgA*	*hecA*	*hecB*	*pldA*	*mviN*	*tlyA*
A. butzleri	Chicken meat/58	90	71	100	53	34	48	93	98	100
	Pork/10	90	60	80	40	10	60	100	90	100
	Beef/11	82	45	100	9	27	36	82	100	100
	Total/79	89	66	97	46	30	48	92	97	100
A. cryaerophilus	Chicken meat/2	0	0	100	0	0	0	0	0	0
	Pork/1	100	0	100	0	0	0	0	0	0
	Beef/3	0	0	100	0	0	0	0	100	100
	Total/6	17	0	100	0	0	0	0	50	50

All *A. cryaerophilus* isolates possessed the *ciaB* gene, while *mviN* and *tlyA* were detected in 3 out of the 6 isolates. Only one isolate carried the *cadF* gene. *cj1349*, *hecA*, *hecB*, *irgA*, and *pldA* genes were not detected in this population. All the 3 beef-derived *A. cryaerophilus* isolates carried *ciaB*, *mviN*, and *tlyA* genes. Two *A. cryaerophilus* isolates from chicken meat harboured *ciaB* gene only. The pork-derived isolate harboured *ciaB* and *cadF* genes (Table 1).

3.2. Genotypes of A. butzleri and A. cryaerophilus. Seventy-four genotypes were distinguished within 79 *A. butzleri* isolates (Figure 1). These isolates could be grouped into 8 clusters using 90% cut-off similarity. Nineteen from 21 isolates derived from beef and pork were found to be closely related to *A. butzleri* from chicken meat. Genotypes of 6 isolates from beef and 6 from pork shared >90% similarity to chicken isolates, when >80% similarity to chicken genotypes was found in 4 isolates from beef and 3 isolates from pork. Only two genotypes, one shared by beef (isolate 204) and one by pork isolate (isolate 60), were found to be distant from chicken genotypes. No relation between *A. butzleri* genotype and incidence of virulence genes could be found.

Each of the 6 *A. cryaerophilus* isolates was found to have unique genotype (Figure 2). Over 80% genotype similarity was only found within two beef and two pork *A. cryaerophilus* isolates but not between isolates from beef, pork, and chicken meat.

4. Discussion

A. butzleri and *A. cryaerophilus* are considered as potential food-borne pathogens [28], but the knowledge on the presence of virulence-associated factors and genetic diversity of food-derived isolates is still limited. Thus, we examined the prevalence of virulence-associated genes in *A. butzleri* and *A. cryaerophilus* populations recovered from retail pork, beef, and chicken meat. The *tlyA*, *cj1349*, *mviN*, *pldA*, *cadF*, and *ciaB* genes were found to occur most frequently in *A. butzleri* isolates. Similar results were obtained by Collado et al. [18], who showed that at least 73% of *A. butzleri* isolates from shellfish in Chile harboured *cadF*, *ciaB*, *mviN*, *pldA*, and *tlyA genes*, although only 31% of the isolates possessed *cj1349* gene. In turn, in Belgium [17], Germany [29], and Iran [21], these six genes were found in all *A. butzleri* isolates recovered from various sources, including meat. A high occurrence of these genes was also reported in Spain, where all isolates from meat, mussels, and sewage possessed *ciaB* gene, when 92% of them harbored *cadF* and *cj1349* genes [30]. Consistent to other reports, we showed *hecA*, *hecB*, and *irgA* genes to occur most rarely within *A. butzleri* population [17, 18, 29, 30].

Our results indicate that 14% of *A. butzleri* isolates harboured all nine virulence-associated genes. These results are similar to that obtained by Douidah et al. [17], who found all these genes in 15% of *A. butzleri* isolates, and Karadas et al. [29], who reported 13% of *A. butzleri* isolates carrying all mentioned virulence-associated genes.

Consistent with previous reports [18, 21, 30], we noted a higher incidence of virulence-associated genes in *A. butzleri* as compared to *A. cryaerophilus*. In our study, only *ciaB* gene was detected in all the six *A. cryaerophilus* isolates, *mviN* and *tlyA* were detected in three isolates, and *cadF* gene was detected in one isolate. A high frequency of virulence genes was observed in beef-derived isolates, since all the three isolates possessed *ciaB*, *mviN*, and *tlyA* genes. A high incidence of *mviN* (100%) and *tlyA* (62%) genes was already reported in *A. cryaerophilus* derived from cattle [21]. Collado et al. [18] showed that *mviN* and *tlyA* genes commonly occur in *A. cryaerophilus* from molluscs, when Douidah et al. [17] reported *mviN* gene to dominate in *A. cryaerophilus* from human and animals. In contrast to *A. butzleri*, no *A. cryaerophilus* isolates harboured multiple virulence genes, which is consistent with results obtained by other authors [17, 18, 21]. According to Douidah et al. [17], this difference could be explained by a different pathogenic behaviour of these species or due to a high heterogeneity of their genomes.

A high diversity of virulence genes pattern was found in *A. butzleri* isolates from each source, except *ciaB* and *irgA* genes found to be more frequent in *A. butzleri* from poultry.

Relatively little is known on genetic structure of *Arcobacter* spp. population and even less on impact of genetic background on distribution of virulence-associated genes. Data obtained in this study demonstrate no relation between genotype and virulence genes repertoire in studied *Arcobacter* species. According to our data, even closely related isolates may carry different virulence genes. Similar observation was already made by Lehmann et al. [19] studying *Arcobacter* spp. genotypes from food in Germany.

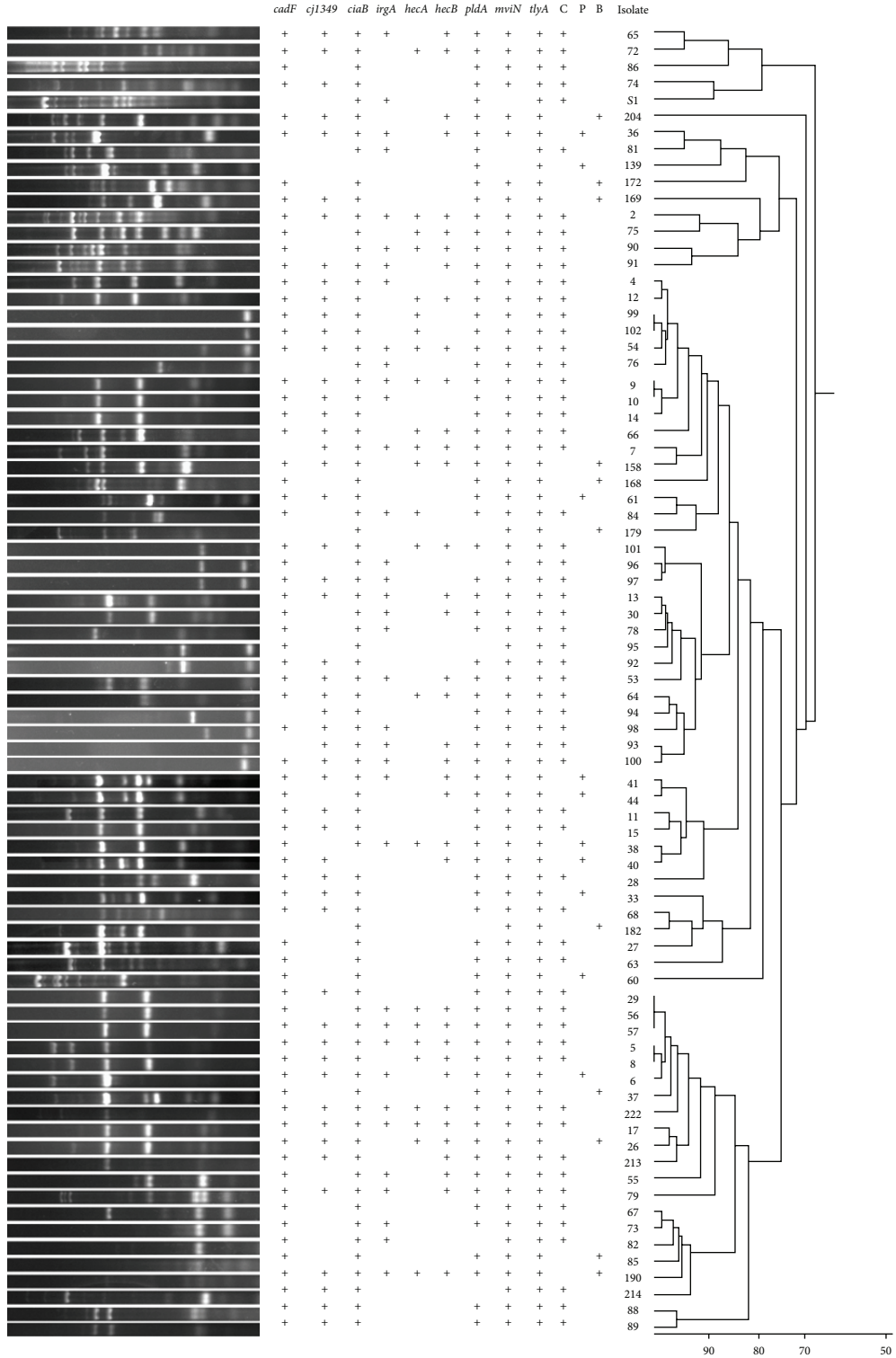

Figure 1: Dendrogram generated by UPGMA clustering based on PCR fingerprinting using ERIC primers performed on 79 *A. butzleri* isolates. Repertoire of virulence-associated genes of each isolate was presented in left panel. Isolate origin was indicated as C, P, and B for chicken, pork, and beef meat, respectively.

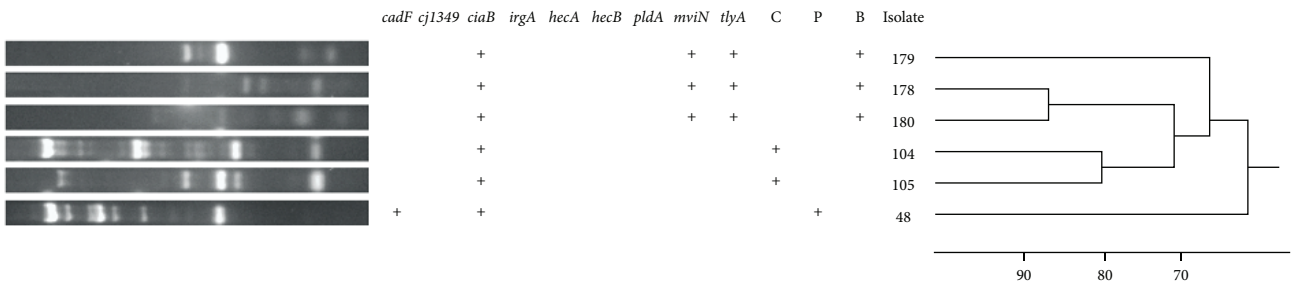

Figure 2: Dendrogram generated by UPGMA clustering based on PCR fingerprinting using ERIC primers performed on 6 *A. cryaerophilus* isolates. Repertoire of virulence-associated genes of each isolate was presented in left panel. Isolate origin was indicated as C, P, and B for chicken, pork, and beef meat, respectively.

Irrespective of genotypic method used, a large genetic heterogeneity of *Arcobacter* spp. population is frequently reported [20, 31–33]. However, some reports point out that closely related *Arcobacter* spp. genotypes can be isolated from some nonoverlapping sources like beef, lamb, pork, poultry, and fish meat [9, 19]. Our data also argue that genotypes closely related to *A. butzleri* from poultry meat can also be found within populations from pork and beef meat. In *A. cryaerophilus* isolates studied here, low genetic relatedness between pork, beef, and chicken meat isolates was noted, but general conclusions cannot be drawn here due to limited number of isolates.

5. Conclusions

Repertoire of virulence-associated genes in *A. butzleri* and *A. cryaerophilus* isolates from retail meat in Poland is similar to that reported in other countries. It seems that closely related genotypes can spread within pork, beef, and chicken meat populations of *A. butzleri* but not *A. cryaerophilus*.

Conflict of Interests

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Research Article

Survival of Unstressed and Acid-, Cold-, and Starvation-Stress-Adapted *Listeria monocytogenes* in Ham Extract with Hops Beta Acids and Consumer Acceptability of HBA on Ready-to-Eat Ham

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The efficacy of hops beta acids (HBA) against unstressed and stress-adapted *Listeria monocytogenes* in ham extract and the consumers' acceptability of HBA on ready-to-eat (RTE) hams were investigated. Unstressed or acid-, cold-, or starvation-stress-adapted *L. monocytogenes* was inoculated (1.3–1.5 log CFU/mL) into 10% ham extract, without (control) or with HBA (4.44 or 10.0 μg/mL). Survival/growth of the pathogen during storage (7.2°C, 26 days) was monitored periodically. Sensory evaluation (30 participants, 9-point hedonic scale) was performed with hams dipped into 0.05, 0.11, and 0.23% HBA solution. Ham extracts without HBA supported rapid growth of unstressed and stress-adapted cells with growth rates of 0.39–0.71 log CFU/mL/day and lag phases of 0–3.26 days. HBA inhibited growth of unstressed *L. monocytogenes* by slowing ($P < 0.05$) growth rate (0.24–0.29 log CFU/mL/day) and increasing ($P < 0.05$) length of the lag phase (3.49–12.98 days) compared to control. Acid-, cold-, or starvation-stress-adapted cells showed cross protection against HBA with greater ($P < 0.05$) growth rates (0.44–0.66 log CFU/mL/day) and similar or shorter lag phases (0–5.44 days) than unstressed cells. HBA did not ($P > 0.05$) affect sensory attributes of RTE ham. These results are useful for RTE meat processors to develop operational protocols using HBA to control *L. monocytogenes*.

1. Introduction

Listeria monocytogenes, a Gram-positive, non-endospore-forming, facultative, and psychrotrophic foodborne pathogen, causes listeriosis, which is an important public health problem in the United States [1]. The groups with high risk to listeriosis include older adults, pregnant women, newborn babies, and immune-compromised patients [2]. The "zero-tolerance" policy was established by the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) in early 1990s for guidance and standards of testing and control of *L. monocytogenes* in ready-to-eat (RTE) meat products [3]. However, from 1998 to 2002, several multistate outbreaks of listeriosis associated with RTE deli meat products occurred in the United States [4–7]. Immediately after these outbreaks, in 2003, the USDA-FSIS began to require RTE meat processors to execute "three alternatives" for *L. monocytogenes* control. Alternatives 1 and 2 require the use of postlethality treatments including the application of antimicrobials [8]. However, since then, the detected presence of *L. monocytogenes* in RTE meat and poultry products has decreased gradually. Contamination of *L. monocytogenes* in deli meat still costs approximately $1.1 billion and 4,000 deaths each year in USA [9]. Therefore, processors of RTE products should continue to develop effective approaches for control of *Listeria* during RTE meat processing [9].

Various stresses adapted cells often develop and survive in the meat processing environment or on meat surfaces. For instance, increasing use of acid treatments, such as lactic or acetic acids, on beef or poultry carcasses induced

the development of acid-stress-adapted pathogenic cells. Similarly, low temperature food storage may lead to cold-stress-resistant cells; exposure of cells to poor nutrition areas such as facility surfaces, walls, and floors may induce starvation-stressed cells [10]. The efficacy of antimicrobials is sometimes decreased due to the generation of cross protection when a stress-adapted cell is subsequently exposed to a sequentially sublethal stress [11]. Therefore, the stress response of foodborne pathogens in different food systems has recently received much research attention.

Hops beta acids (HBA), extracted from hops flowers, with primary components of lupulone ($C_{27}H_{38}O_4$), colupulone ($C_{26}H_{37}O_4$), and adlupulone ($C_{27}H_{38}O_4$) have been approved by the USDA-FSIS and US-Food and Drug Administration to be used as generally recognized as safe (GRAS) antimicrobial agents on cooked meat surfaces and in casings [12, 13]. Previous studies by Shen and Sofos showed that HBA can efficiently inhibit growth of *L. monocytogenes* in a culture broth medium [14] and on frankfurters under vacuum packaged storage [15]. However, no published literature addressed the antilisterial activity of HBA in food systems, particularly with various stresses adapted *L. monocytogenes*. In addition, hops have been well known for a special dark brownish color and bitter taste that contribute to the beer brewing process, and no published studies evaluated consumer acceptability of application of HBA on RTE meat products.

Therefore, the objectives of this study were to evaluate the efficacy of HBA to inactivate unstressed and acid-, cold-, and starvation-stress-adapted *L. monocytogenes* in ham extract during storage at 7.2°C and the sensory acceptability of HBA applied on commercial RTE hams.

2. Materials and Methods

2.1. Bacterial Strains and Preparation of Unstressed or Stress-Adapted Cells. The 4-strain mixture of *L. monocytogenes* (kindly provided by Dr. Joshua Gurlter, at USDA-ARS, Wyndmoor, PA) used in this study included ATCC 15213, Scott A 724 (Massachusetts meat outbreak, serotype 4b), L499 (sliced turkey outbreak strain, human isolate, serotype 1/2a), and L502 (chocolate milk outbreak, serotype 1/2b). Each *L. monocytogenes* strain, taken from the −20°C stock culture, was first activated by streak plating onto PALCAM agar (Difco, BD, Sparks, MD) and then incubated at 35°C for 48 h. The procedure of preparing unstressed and three types of stress-adapted cells followed the previous study [16]. To prepare the unstressed cells, a single colony of each *L. monocytogenes* strain was cultured and subcultured (35°C, 24 h) in 10 mL of glucose-free tryptic soy broth with yeast extract (TSB-G + YE). To prepare the acid-stress-adapted cells, the TSB-G + YE cultured (35°C, 24 h) cell suspension was subcultured (0.1 mL, 24 h at 35°C) into 10 mL of TSB-G + YE supplemented with 1% glucose. For preparation of cold- and starvation-stress-adapted cells, the TSB-G + YE cultured (35°C, 24 h) cell suspension was first triplicate-washed in 10 mL phosphate-buffered saline (PBS, pH 7.4), resuspended in 10 mL of TSB-G + YE with storage at 4°C for 7 days, and resuspended in 10 mL of 0.85% NaCl solution with storage at 35°C for 48 hours, respectively. Prior to the experiment, the unstressed or acid-, cold-, or starvation-stress-adapted cells of each strain were washed by centrifuging at 4,629 ×g for 15 min at 4°C three times with 10 mL PBS. The cell pellets were resuspended and serially diluted in PBS to reach a target inoculation level of 1.3 to 1.6 log CFU/mL when 0.1 mL of inoculum was added into ham extract solutions.

2.2. Ham Extract Preparation and Inoculation. Fresh uncured ham was purchased from a local supermarket at Wuxi, Jiangsu, China, and manually cut into 7 × 8 cm^2 pieces. The 10% (w/w) ham extract was prepared by placing the cut ham pieces into distilled water (1 : 10 by volume), homogenized for 2 min in a masticator (IUL Instruments, Barcelona, Spain), and then passed through 2 layers of cheese cloth. The homogenate was autoclaved to sterilize natural bacterial flora and cooled to room temperature before aseptically dispensing 100 mL into sterile glass bottles. HBA solution (45% product, brownish purple color, water soluble, density: 1.07 ± 0.01 g/mL), kindly provided by S.S. Steiner Inc. at New York, NY, was dissolved in distilled water and added to the aforementioned sterile ham extract in appropriate amounts to reach concentrations of 0, 4.44 and 10 mg/L. As previously indicated, the 100 mL of ham extract solutions was inoculated with 0.1 mL of the diluted inoculum. The inoculated glass bottles were then stored at a refrigerated incubator set at 7.2°C (Fisher Scientific, Fair Lawn, NY, stability ±0.2°C) for up to 26 days.

2.3. Microbiological and pH Analyses. On days 0, 3, 6, 9, 12, 16, 20, and 26 during storage, an aliquot of 5 mL solution for each treatment was 10-fold serially diluted in 0.1% peptone water and surface plated onto tryptic soy agar (Difco, Becton Dickinson), supplemented with 0.6% yeast extract (Acumedia, Lansing, MI; TSAYE) and PALCAM agar for enumeration of *L. monocytogenes* in a support medium and a selective medium, respectively. Colonies were counted manually after incubation at 30°C for 48 h with a detection limit of 0.5 log CFU/mL. Following microbial analysis, the pH of the homogenate was measured using a digital pH meter (Fisher Scientific, Fair Lawn, NY).

2.4. Sensory Evaluation. Sensory analysis was performed by evaluating consumer acceptability of unheated, fresh purchased RTE ham after dipping into HBA solutions. The fresh purchased ham was sliced in a Hobart 2712 12″ semiautomatic slicer (Hobart Mfg. Co., Troy, OH) and manually cut into pieces of 7 cm × 8 cm per side with total surface area of 112 cm^2. The ham slices were left untreated (control) or were immersed in 0 (distilled water), 0.05, 0.11, and 0.23% HBA solutions to reach the residual HBA concentrations of 0, 2.0, 4.44, and 10 mg/kg on the product surface. The dipping treatment was applied by immersing 20 pieces of ham in 250 mL of prepared HBA solution for 2 min, followed by draining for 1 min, vacuum packaging (A300/16, Multivac Inc., Germany), and overnight storage at 4°C. To verify absorption of HBA on the surface of ham (mg/kg), preliminary experiments were conducted to determine the weight gained by each ham

piece after 2 min of dipping into distilled water followed by draining for 1 min as described in the previous study [15].

The sensory evaluation tests were approved by the Jiang Nan University Institutional Review Board (IRB) and were conducted in a state-of-the-art sensory laboratory. A random coded three-digit number was assigned to each sample to identify treatment groups. An untrained panel of 30 consumers was recruited from the School of Food Science and Technology at Jiang Nan University (Wuxi, Jiangsu, China) to evaluate hams for appearance, color, odor, flavor, texture, and overall acceptability. Room temperature water in plastic cups and fresh unsalted crackers were provided to each panel member to clean their palates well between samples. A 9-point hedonic scale, where 1 indicates dislike extremely and 9 indicates like extremely, was used to evaluate the appearance, odor, flavor, and overall acceptability of hams. The color (1 indicating extremely pale and 9 indicating extremely dark) and texture (1 indicating extremely soft and 9 indicating extremely firm) of hams were also evaluated.

2.5. Statistical Analysis and Data Modeling. The experiment was performed twice, and for each replication three individual samples were analyzed at each sampling time ($n = 6$). The pH and microbiological data (converted to log CFU/mL) were analyzed using the Mixed Model Procedure of SAS with independent variables including type of stress, treatment, time, and interactions between two and three independent factors. Results of sensory evaluation were analyzed using One-Way ANOVA of SAS. Means and standard deviations were calculated, and the differences among subgroup means were separated using a LSD adjustment for multiple comparison at the significance level of $\alpha = 0.05$. USDA-Integrated-Predictive-Modeling-Program (IPMP) [17] and DMFIT software (Institute of Food Research, Reading, UK) were used to estimate parameters of the pathogen cells' survival/growth curve during storage. For each model, the six repeats of experimental data were used to estimate, through the root mean square error (RMSE) and Akaike Information Criterion (AIC), how well the model predicted the data.

3. Results and Discussion

3.1. Survival/Growth of Unstressed or Stress-Adapted L. monocytogenes in Ham Extract after Exposure to HBA. Recently, the U.S. National Advisory Committee on Microbial Criteria for Foods suggested that the evaluation of antimicrobial agents inhibiting *L. monocytogenes* growth on RTE meat products should include a temperature of 45°F (7.2°C), which reflects the real RTE meat processing environment [9]. In this study, the growth behavior of unstressed or acid-, cold-, or starvation-stress-adapted *L. monocytogenes* in ham extract containing 4.44 or 10.0 mg/L of HBA was evaluated during storage at 7.2 ± 0.2°C. Throughout the 26 days of storage, the *L. monocytogenes* populations for all treatments on TSAYE (Figure 1) did not differ ($P > 0.05$) from those observed on PALCAM agar (Figure 2), indicating that the majority of *L. monocytogenes* can recover and grow on selective agar and HBA did not cause injury of the pathogen cells [18]. Therefore, the *L. monocytogenes* populations derived from the PALCAM agar were used to describe the growth dynamics of all treatments.

The initial unstressed and acid-, cold-, and starvation-stressed *L. monocytogenes* populations were 1.7, 1.5, 1.7, and 1.3 log CFU/mL, respectively. As expected, the unstressed and 3 types of stress-adapted pathogen cells grew rapidly and reached 7.8 to 8.7 log CFU/mL by the end of storage (Figure 2). At day 26, the final population of acid-, cold- and starvation-stressed cells averaged 0.8 log CFU/mL greater ($P < 0.05$) than that of the unstressed cells (Figure 2).

After exposure to HBA, the survival/growth behavior of unstressed and 3 types of stress-adapted cells was different ($P < 0.05$). For unstressed pathogenic cells, no immediate reduction was observed in 4.44 and 10 mg/L HBA solutions (Figure 2), which agrees with previous studies by Shen and Sofos [14], who reported that the initial amounts of *L. monocytogenes* in all treatments with or without HBA ranged from 2.6 to 2.8 log CFU/mL. During storage at 7.2°C, the unstressed *L. monocytogenes* growth was inhibited by HBA and this inhibition increased with increasing concentrations of HBA, which is in agreement with previous studies by Shen and Sofos [14] and Shen et al. [15]. The inhibition continued for up to 6 days and 12 days for 4.44 and 10 mg/L of HBA, resulting in 0.6 ($P > 0.05$, 4.44 mg/L of HBA) and 3.3 log CFU/mL ($P < 0.05$, 10.0 mg/L of HBA) lower pathogen populations compared to the control by the end of 26-day storage (Figure 2). The mode-of-action of HBA for the inhibition of *L. monocytogenes* growth is attributed mainly to the leakage of the cytoplasmic contents, the release of protons with a proton motive force depletion, the drop of intracellular pH, and the inhibition of the active transport of sugar and amino acids [19].

In general, after exposure to 4.44 or 10.0 mg/L of HBA, the acid-, cold- and starvation-stress-adapted *L. monocytogenes* cells showed fast growth and high final pathogen populations, ranging from 8.3 to 8.4, 8.5 to 8.8, and 8.6 to 8.9, respectively (Figure 2). Among the three types of stress-adapted cells, the growth curves of 4.44 mg/L HBA-treated samples were very similar to the control (Figure 2), which shows an apparent loss of inhibitory activity of 4.44 mg/L HBA to various stressed cells. During storage, a lower ($P < 0.05$) pathogen growth was noticed in 10.0 mg/L HBA-treated samples than in those from the control, particularly for cold- and starvation-stressed cells. However, this inhibition was much lower ($P < 0.05$) than that observed in unstressed cells. Therefore, a cross protection effect obviously developed among acid-, cold- or starvation-stress-adapted cells when exposed to HBA.

The strategies used by microorganisms to resist acid stress include pH homeostasis, changes in membrane structure by alteration of protein permeability, internal buffering ability, and the pH stability of essential proteins [20]. To survive in a cold-stress environment, bacterial cells usually modify the cell membrane to maintain membrane fluidity and macromolecular structural integrity in proteins and ribosomes [21, 22] and synthesize cold shock proteins [23]. Starvation stress causes an increase in cellular resistance capability by means of the use of alternative growth substrates or energy

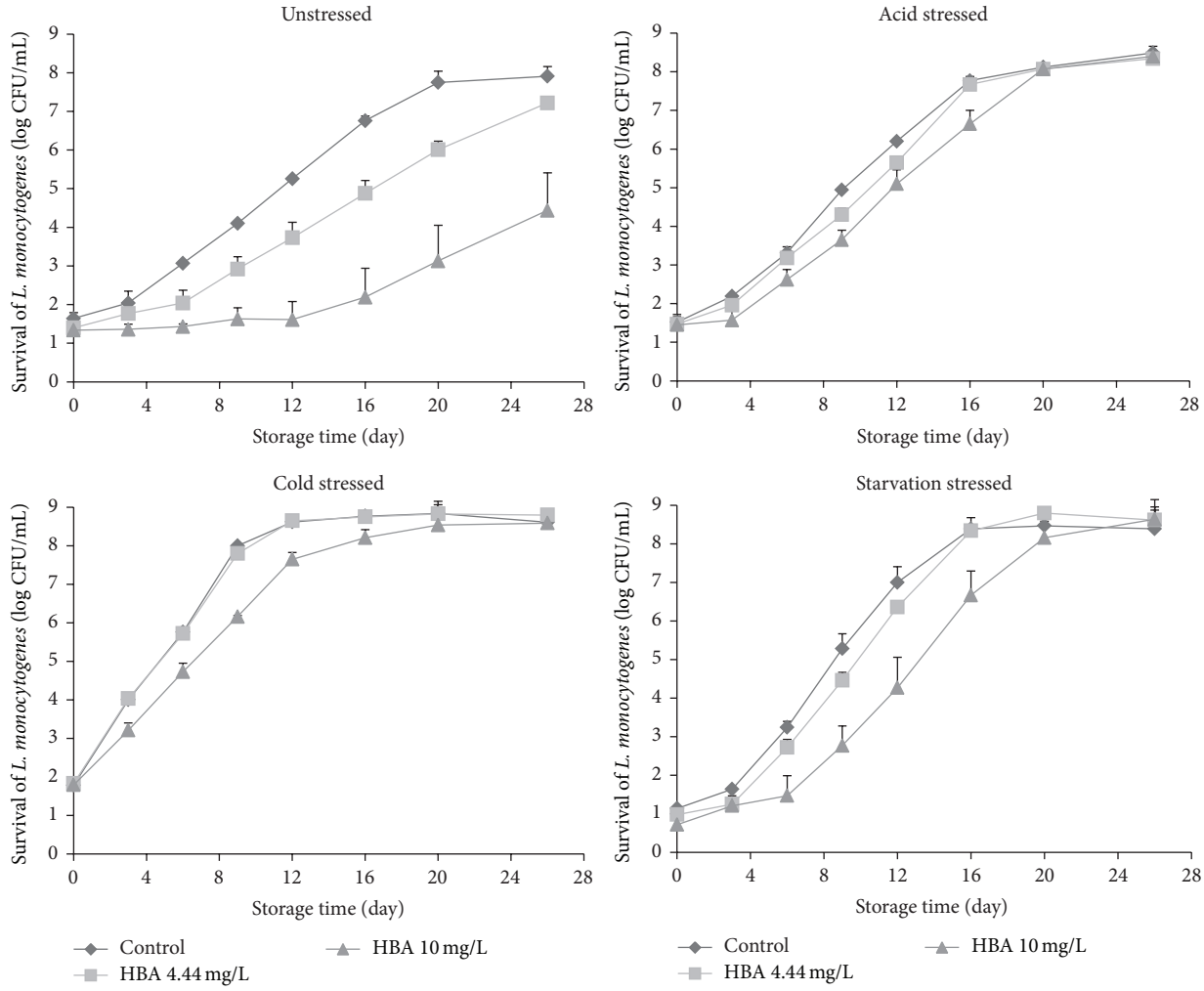

Figure 1: Means (SD, log CFU/mL) of unstressed and acid-, cold-, and starvation-stressed *L. monocytogenes* (tryptic soy agar with 0.6% yeast extract) populations in ham extract containing 0.0 (control), 4.44, and 10.0 mg/L hops beta acids (HBA) during storage at 7.2°C for 26 days.

to stabilize ribosomes against degradation [24], change morphological transformation into spherical conformations [25], and enhance metabolic potential of microorganisms [26]. It is reported that starvation proteins are encoded by two groups of genes, including *cst* genes controlled by carbon starvation and *pex* genes controlled by carbon, nitrogen, or phosphorus starvation [26].

3.2. Data Modeling. In a preliminary study, four reduced and four full growth models in the USDA-IPMP software were used to evaluate the fitness of the model to predict the growth kinetics of cells in different treatments (i.e., low value of RMSE and AIC). As shown in Table 1, the Baranyi Full Growth Model fitted well growth data for all treatments based on their low RMSE (from 0.152 to 0.524) and low AIC scores (from −12.545 to −99.284). Therefore, the DMFIT software, based on Baranyi Full Growth Model, was used to compare the lag phase periods and growth rates in all treatments (Table 2).

For unstressed *L. monocytogenes* cells, reduced growth rates of 0.29 and 0.24 log CFU/mL/day were obtained in 4.44 and 10.0 mg/L HBA-treated ham extract (Table 2), respectively, which were lower ($P < 0.05$) than in ham extracts without HBA (control, growth rates of 0.39 log CFU/mL/day, Table 2). Corresponding to the microbiological data (Figures 1 and 2), HBA treatments extended the lag phase time from 2.49 days in controls to 3.49 ($P > 0.05$) and to 12.98 days ($P < 0.05$) in 4.44 and 10.0 mg/L HBA treatments, respectively (Table 2). These findings verified that HBA had promising bacteriostatic effects for unstressed *L. monocytogenes* cells in ham extract.

In the absence of HBA, the three types of stress-adapted cells behaved differently ($P < 0.05$) during storage with the calculated growth rates increasing as 0.39 (unstressed) < 0.50 (acid) < 0.68 (starvation) ≤ 0.71 (cold) log CFU/mL/day and lag phase periods decreasing as 3.26 (starvation) ≥ 2.49 (unstressed) ≥ 2.33 (acid) > undetected (cold) (Table 2). The longer lag phase time shown in starvation-stressed

Table 1: Comparison of RMSE and AIC for the proposed models fitting the growth of unstressed or acid-, cold-, and starvation-stress-adapted *Listeria monocytogenes* in the presence of hops beta acids (HBA) in ham extract.

Stress types	HBA (mg/L)		Reduced growth model				Full growth model			
			No lag phase	Reduced Huang	Reduced Barany	Buchanan two-phase linear	Huang	Baranyi	Modified Gompertz	Buchanan three-phase linear
Unstressed	0	RMSE	0.346	0.593	0.580	0.593	0.234	0.238	0.265	0.211
		AIC	−44.214	−18.300	−19.320	−18.300	−61.009	−60.207	−55.032	−66.168
	4.44	RMSE	—	0.299	0.297	0.300	0.290	0.291	0.290	—
		AIC	—	−51.095	−48.294	−51.022	−50.866	−50.623	−50.713	—
	10.0	RMSE	—	0.629	0.635	0.629	—	0.643	—	—
		AIC	—	−15.486	−15.008	−15.474	—	−12.545	—	—
Acid	0	RMSE	0.291	—	0.738	0.804	0.178	0.152	0.184	1.161
		AIC	−52.482	—	−7.813	−3.679	−74.253	−81.857	−72.522	15.801
	4.44	RMSE	0.321	—	0.679	0.708	0.209	0.176	0.245	0.169
		AIC	−47.684	—	−11.814	−9.806	−66.629	−74.901	−58.848	−76.699
	10.0	RMSE	0.469	0.581	0.603	0.581	0.302	0.313	0.330	—
		AIC	−29.520	−19.302	−17.465	−19.306	−48.834	−47.104	−44.580	—
Cold	0	RMSE	0.158	—	0.918	1.489	—	0.162	0.249	0.691
		AIC	−81.759	—	2.708	25.898	—	−78.793	−58.080	−9.139
	4.44	RMSE	0.108	—	0.848	1.379	—	0.106	0.218	0.736
		AIC	−100.221	—	−1.093	22.216	—	−99.284	−64.410	−6.110
	10.0	RMSE	0.209	—	0.777	1.053	0.208	0.206	0.258	0.892
		AIC	−68.320	—	−5.309	9.263	−66.632	−67.189	−56.481	3.147
Starvation	0	RMSE	0.544	—	1.103	1.174	0.354	0.343	0.365	0.357
		AIC	−22.455	—	11.520	14.489	−41.242	−42.801	−39.817	−40.759
	4.44	RMSE	0.464	—	1.012	1.043	0.216	0.189	0.251	0.263
		AIC	−30.056	—	7.391	8.824	−64.852	−71.459	−57.782	−55.544
	10.0	RMSE	0.792	0.815	0.843	0.815	0.523	0.524	0.529	0.544
		AIC	−4.420	−3.016	−1.396	−3.004	−22.488	−22.429	−21.937	−20.591

Note. RMSE: root mean sum of squared errors; AIC: Akaike Information Criterion (the smaller the better); "—": model is unlikely for the data.

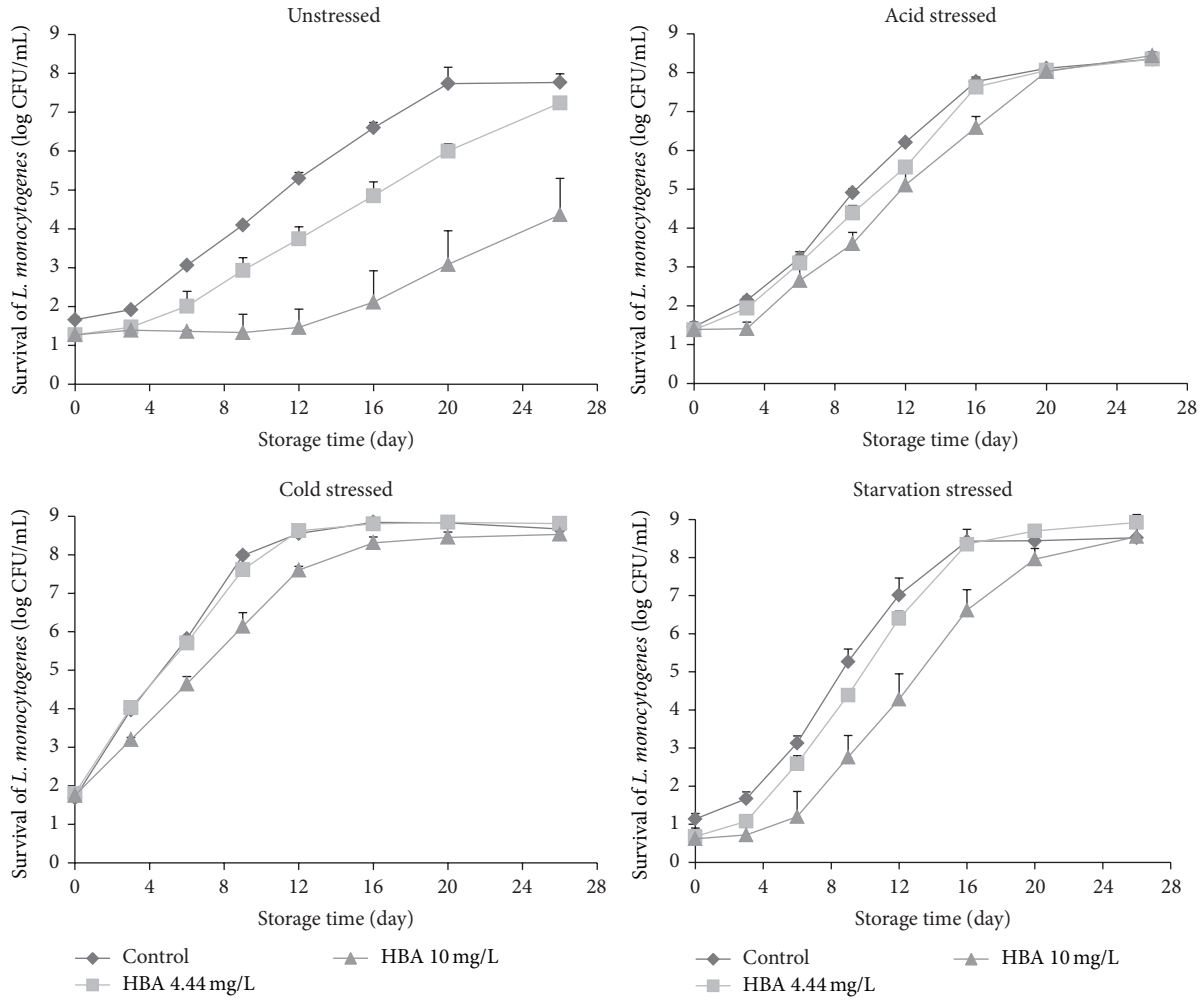

Figure 2: Means (SD, log CFU/mL) of unstressed and acid-, cold-, and starvation-stressed *L. monocytogenes* (PALCAM agar) populations in ham extract containing 0.0 (control), 4.44, and 10.0 mg/L hops beta acids (HBA) during storage at 7.2°C for 26 days.

Table 2: Means (S.E.) of lag phase duration (LPD) and growth rate (GR) of unstressed and acid-, cold-, and starvation-stress-adapted *L. monocytogenes* during exposure to hops beta acids (HBA) in ham extract calculated using the Baranyi Full Growth Model in DMFIT software.

Type of stress	HBA concentration	LPD (days)	GR (log CFU/ml/day)	R^2
Unstressed	0.0 mg/L (control)	2.49[a] (0.79)	0.39[a] (0.03)	0.99
	4.44 mg/L	3.49[a] (1.30)	0.29[b] (0.03)	0.98
	10.0 mg/L	12.98[b] (3.14)	0.24[b] (0.07)	0.93
Acid	0.0 mg/L (control)	2.33[a] (0.46)	0.50[c] (0.02)	0.99
	4.44 mg/L	2.89[a] (0.46)	0.48[c] (0.02)	0.99
	10.0 mg/L	3.42[a] (0.88)	0.44[c] (0.03)	0.99
Cold	0.0 mg/L (control)	—	0.71[d] (0.02)	0.99
	4.44 mg/L	—	0.65[d] (0.01)	0.99
	10.0 mg/L	—	0.50[c] (0.01)	0.99
Starvation	0.0 mg/L (control)	3.26[a] (0.73)	0.68[d] (0.07)	0.99
	4.44 mg/L HBA	3.30[a] (0.40)	0.66[d] (0.03)	0.99
	10.0 mg/L HBA	5.44[a] (1.13)	0.57[c] (0.07)	0.97

—: Lag phase duration is not detected.

[a–d]Means in the same column with the same superscript letter were not significantly different ($P > 0.05$).

Table 3: Sensory analysis of ready-to-eat (RTE) ham treated with hops beta acids (HBA) solution.

Treatment[a]	Appearance[b]	Color[c]	Odor[b]	Flavor[b]	Texture[d]	Overall acceptability[b]
Control	6.25 ± 0.66[a]	5.94 ± 1.03[a]	5.68 ± 1.02[a]	6.28 ± 1.28[a]	5.73 ± 1.37[a]	6.20 ± 1.12[a]
DW	5.99 ± 0.71[ab]	5.21 ± 0.86[a]	5.26 ± 0.89[ab]	5.66 ± 1.39[a]	5.10 ± 0.80[a]	6.14 ± 0.93[a]
0.05% HBA	5.90 ± 0.58[ab]	5.11 ± 0.78[a]	5.33 ± 0.84[ab]	5.46 ± 1.13[a]	6.01 ± 0.82[a]	5.64 ± 1.36[a]
0.11% HBA	6.03 ± 0.82[ab]	5.19 ± 0.56[a]	5.14 ± 0.67[ab]	5.48 ± 1.15[a]	6.06 ± 0.80[a]	5.86 ± 0.86[a]
0.23% HBA	5.24 ± 0.77[b]	4.85 ± 0.85[a]	4.71 ± 0.57[b]	4.58 ± 0.92[a]	5.33 ± 0.80[a]	4.51 ± 1.01[a]

[a]Means with the same letter were not significantly different ($P > 0.05$).
[b]1 = dislike extremely; 9 = like extremely.
[c]1 = extremely pale; 9 = extremely dark.
[d]1 = extremely soft; 9 = extremely firm.

cells can be explained by the "shift-up" effect [27]. After transferring *L. monocytogenes* cells from 0.85% salt solution (starvation stress) to the ham extract, the cells needed extra time to construct new ribosome to enhance their ability for protein synthesis, resulting in a longer lag phase time [27]. Overall, after exposure to HBA, compared to the unstressed *L. monocytogenes* cells, acid-, cold- or starvation-stress-adapted pathogen cells showed higher ($P < 0.05$) growth rates, indicating cross protection effects. Specifically, among the 4.44 and 10.0 mg/L HBA treatments, the growth rates increased as 0.24 to 0.29 (unstressed) < 0.44 to 0.48 (acid) < 0.50 to 0.65 (cold) ≤ 0.57 to 0.66 (starvation) log CFU/mL/day (Table 2). The lag phase periods were similar ($P > 0.05$) in most HBA treatments regardless of various types of stress (Table 2). There was an inhibitory function of HBA on stress-adapted cells, especially for 10.0 mg/L HBA. For example, the growth rates of 10.0 mg/L HBA-treated cold- and starvation-stressed cells (0.50 and 0.57 log CFU/mL/day) were significantly lower than those from the control and 4.0 mg/L HBA treatment.

3.3. pH Variation of Ham Extract. The average initial pH of untreated ham extract solution on day 0 was 6.16, while after adding HBA, the pH slightly increased to 6.21 to 6.24 (Figure 3), which is in agreement with the previous study [14]. As expected, the pH of the ham extract solution during 26 days of storage decreased significantly in samples in which significant growth (>6-7 log CFU/mL) of the unstressed or stress-adapted *L. monocytogenes* occurred in ham extract solution. The pH decrease was attributed to the microbial metabolism of carbohydrates of ham extract, generating acid into ham extract solutions in which significant growth of the pathogen population was observed [18]. For unstressed pathogen cells, the pH of HBA-treated samples did not change significantly ($P \geq 0.05$) until 20 to 26 days of storage (Figure 3), suggesting that the unstressed *L. monocytogenes* did not grow rapidly in the presence of HBA. However, a dramatic decrease of pH value occurred at days 16 to 20, 9 to 16, and 16 for acid-, cold-, and starvation-stress-adapted-cells, respectively, regardless of control or HBA treatments (Figure 3), indicating rapid pathogen growth due to their resistance to HBA.

3.4. Sensory Evaluation. In an early study reported by Shen et al. [15], dipping frankfurters into 0.06 to 0.10% HBA solutions inhibited *L. monocytogenes* for 30 to 50 days of vacuum sealed storage at 4 or 10°C; however, it raised the concern that applying HBA on RTE meats might cause adverse sensory effects. Therefore, in this study, sensory evaluation was performed on unheated RTE hams dipped into 0.05 to 0.23% HBA followed by 24-h vacuum package storage at 4°C. The 30 participating panelists were primarily university food science graduate students (90%) with the age from 21 to 30 years (82%), 55% of whom were male. More than half of the participants (65%) indicated that they liked to eat RTE deli meats and frankfurters, and 50% and 45% of them ate RTE meats one to three times per month and one to five times per year, respectively. The slightly greater standard deviations that ranged from 0.56 to 1.39 (Table 3) were expected in this study, because an untrained consumer style panel was used to perform the sensory evaluation analysis.

The average hedonic scores of untreated ham were 5.68–6.28 across items of appearance, color, odor, flavor, texture, and overall acceptability (Table 3). After dipping into distilled water or 0.05 or 0.11% HBA, slightly ($P > 0.05$) lower scores were obtained among 30 panel members compared to those obtained from the untreated samples. In general, immersing ham in 0.05 and 0.11% HBA did not cause any negative effects on the tested sensory attributes, ranking between "neither like nor dislike" and "like slightly" (Table 3). Color scores were only slightly lower ($P > 0.05$), from 5.11 to 5.19, in 0.05 and 0.11% HBA-dipped hams; thus HBA did not bring severe brownish color to the ham surfaces. However, the lowest mean hedonic scores of all sensory attributes were seen in the 0.23% HBA-treated samples (Table 3). HBA is derived from hops flowers used for beer brewing process and therefore has the potential to impart a "beer like" bitter taste. Adding too much HBA into postlethality dipping solutions may decrease sensory acceptability of treated RTE meat products. In a previous study, Geornaras et al. [28] found that commercial ham and frankfurters treated with organic acids such as lactic and acetic acids resulted in significant lower hedonic scores compared to untreated controls. In this study, 0.05 and 0.11% HBA-treated samples had only slightly lower sensory scores in all attributes. Therefore, it is suggested that HBA did not cause extra adverse sensory effects as compared to the other

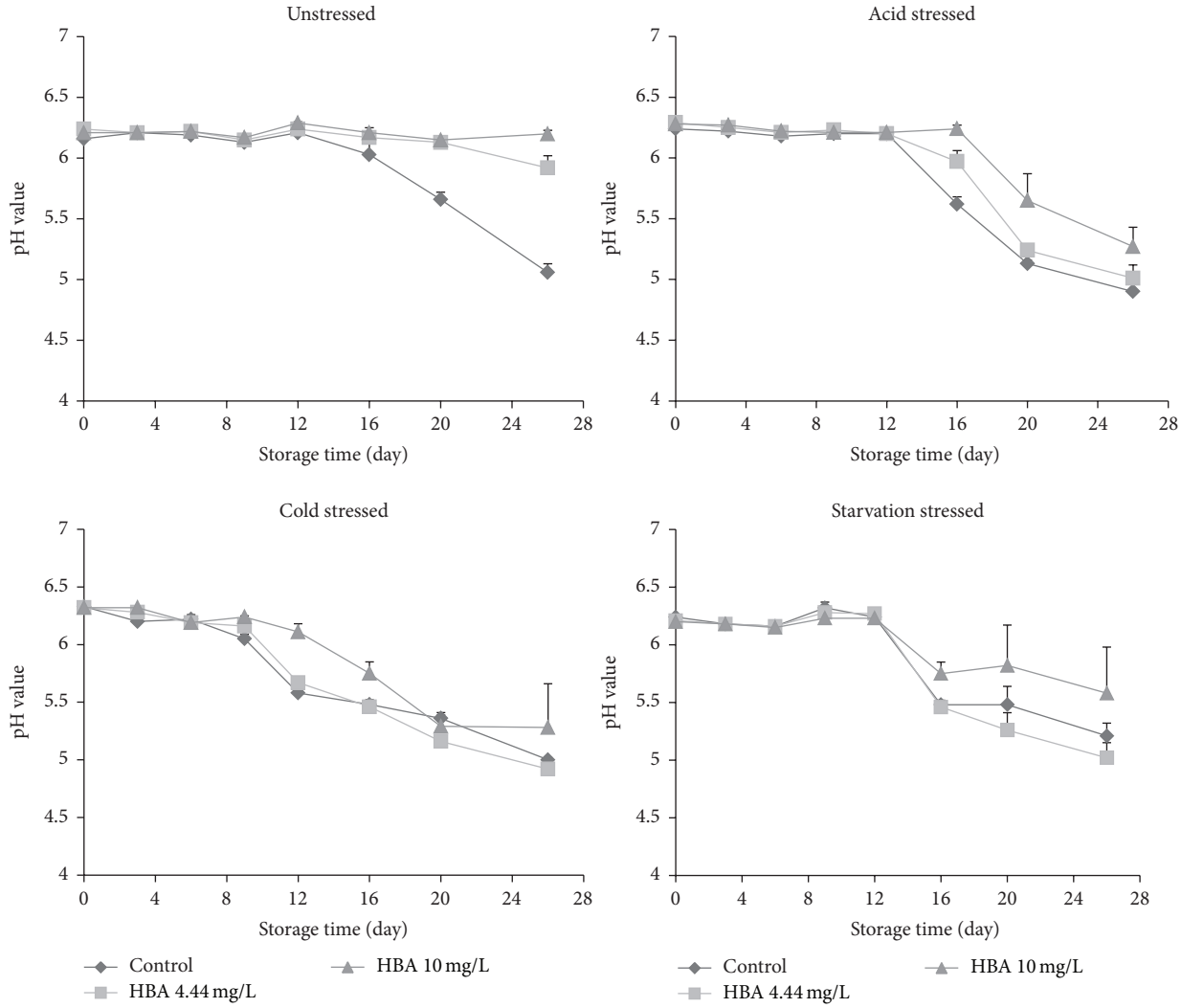

Figure 3: Mean (SD) pH values of ham extract containing 0.0 (control), 4.44, and 10.0 mg/L hops beta acids (HBA) during storage at 7.2°C for 26 days.

widely used organic acid base antimicrobials. Thus, 0.11% HBA, which generated a residual concentration of 4.44 mg/kg HBA on the RTE meat surfaces (recommended by USDA-FSIS), should be confidently applied during postlethality RTE meat processing.

4. Conclusion

Results of the present study indicated that HBA exhibited promising inhibitory effects for unstressed *L. monocytogenes* in ham extract stored at 7.2°C. The sensory evaluation results confirmed that applying 4.4 mg/kg of HBA on RTE hams (approved by USDA-FSIS and US-FDA) will not cause adverse sensory effects. However, acid-, cold-, and starvation-stress-adapted *L. monocytogenes* cells showed resistance and cross protection to HBA. For industrial RTE meat processers, challenge studies should examine whether meat decontamination with organic acids or cold storage may provide cross protection of *L. monocytogenes* to subsequent exposure to antimicrobials, such as HBA, during postlethality processing of RTE meats. Future studies are needed to evaluate the antilisterial activities of HBA on more RTE products such as uncured or cured ham, turkey breast, and roast beef.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Management of Food-Related Diarrhea Outbreak in the Emergency Department: Lessons Learned from the German STEC O104:H4 Epidemic

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Emergency department (ED) management of the German STEC O104:H4 outbreak in 2011 was not limited to patients being truly infected with STEC. In parallel to spread of alarming news in public media, patients suffering from diarrhea due to other reasons fearfully presented, equally. We retrospectively characterized these two cohorts for anamnestic, clinical, and laboratory findings at their first ED contact. From 15th of May to July 2011, 302 adult patients with diarrheal complaint presented at the EDs of two tertiary hospitals in Lubeck, northern Germany. Fecal testing for STEC was obtained in 245 (81%) patients: 105 were STEC-positive and 140 were STEC-negative. Anamnestic characteristics (defecation rate, visible bloody diarrhea, and lower abdominal pain), abdominal tenderness, and some laboratory findings were significantly different between both cohorts but not reliable to exclude STEC. In >90% of STEC-positive patients diarrheal symptoms had started in May, reflecting the retrospective nationwide peak of infections, whereas the majority of STEC-negative patients became symptomatic in June 2011. During the German STEC O104:H4 outbreak a definite distinction at initial ED contact between STEC-positive versus STEC-negative patients by clinical judgment alone was not reliable. Fecal testing in the ED, however, might survey the outbreak of foodborne infections with the utmost precision.

1. Introduction

In 2011, a large outbreak of Shiga toxin-producing *Escherichia coli* (STEC O104:H4) has caused 3816 documented infections in Germany, including 845 confirmed cases of hemolytic uremic syndrome (HUS). According to retrospective epidemiologic analyses of the Robert-Koch Institute (RKI), which is the leading national German health authority, about 90% of diarrheal cases occurred during the second half of May (Figure 1(b)) [1]. During the outbreak period, however, this clear-cut epidemiologic frame was unknown. The first official statement about the outbreak was given on May 20th [2], and an official declaration that the epidemic had ended was published on 26th of July. Likewise disease communication in public media (TV news, press coverage, etc.) continued until July 2011 including alarming reports that reinforced uneasiness in the population.

Emergency departments (EDs) at tertiary hospitals offer service 24 h/7 d. Therefore EDs are prone to a multitude of first medical contacts during outbreaks with foodborne diarrheal pathogens. A rapidly acting network between EDs and health authorities might significantly improve infection

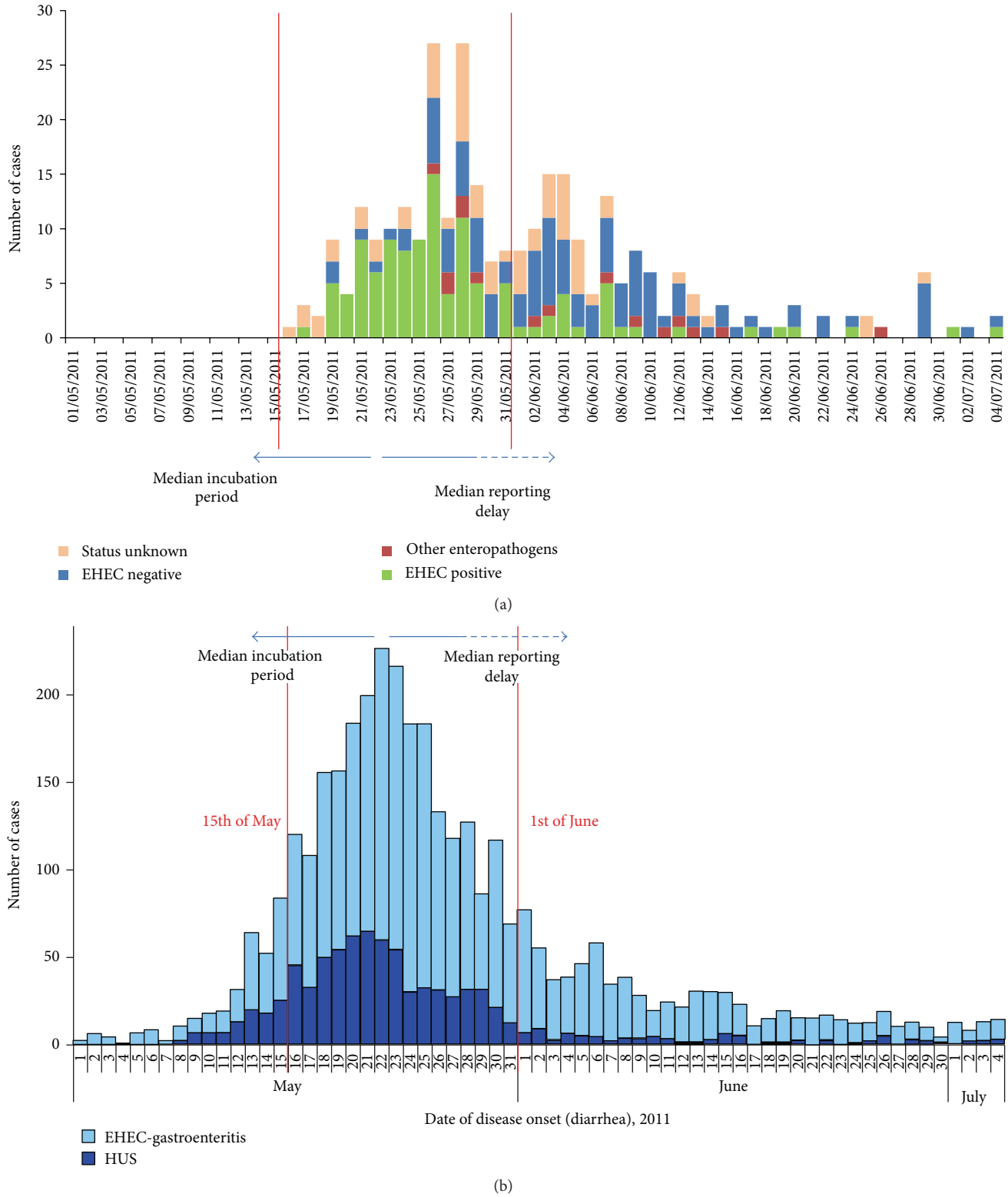

Figure 1: (a) Date and number of patients presenting at the ED in Lubeck with suspected EHEC-infection. The group was subdivided into patients with approved STEC-infection, patients with stool enteropathogens other than STEC, and patients without any fecal pathogens and those who have no valid STEC-result. (b) Nation-wide epidemiology of diarrhea onset in patients with approved STEC-infection with or without hemolytic uremic syndrome (HUS). Peak of diarrhea onset was at 21st of May; arrow to the left side indicates median incubation period of eight days and arrow to the right side indicates median reporting delay of > one week (adapted from [1]).

surveillance and accelerate outbreak control. In the context of STEC-outbreaks the special challenge for the ED is to detect and manage (a) STEC-infected patients presenting with variable clinical manifestations, (b) to distinguish patients suffering from diarrhea due to other infectious or noninfectious reasons, and (c) to screen ED presenters driven by anxiety rather than objective clinical findings. This resource-demanding aspect is neglected in epidemiologic reports and most scientific work-up of the German STEC/HUS-outbreak 2011, though it was of high, yet undetermined social and health-economic impact.

During the outbreak a definite distinction at initial ED contact between STEC-infected and noninfected patients by pure clinical judgment was not possible and clinical criteria indicating risk of future HUS-development were unknown. Therefore, stratification for the need of follow-up was not possible until microbiological results of fecal testing were available. Here we report anamnestic, clinical, and laboratory characteristics of STEC-positive compared to STEC-negative outbreak-related ED contacts from May to July 2011 in Luebeck, one of the most affected cities in northern Germany. Almost all patients were subject to standardized anamnestic, clinical, and laboratory assessment and fecal microbiologic testing was initiated. Comparing both cohorts retrospectively we reflect the impact of EDs for infection surveillance and outbreak control.

2. Patients and Methods

During the outbreak period all adult patients were documented who presented to any one of two tertiary hospital EDs in Luebeck, the university hospital (UKSH) and the municipal hospital (Sana-Klinik), with any complaint consistent with STEC-infection including diarrheal symptoms, visible blood in stool, "bleeding hemorrhoids," or personal fear of being infected. The first official press release about the outbreak was given by the RKI on May 20th [2]. Starting from 21st of May history taking was standardized with a questionnaire that was filled in by the patients and/or the ED doctors. This included the following questions: beginning of diarrheal symptoms, estimated number of (bloody) stools within last 24 h, upper/lower/diffuse abdominal pain, fever, nausea and vomiting, alimentary details, and history of travel.

Stool specimen sampling was rigorously performed since 20th of May 2011 using standard methods for *E. coli* culture and Shiga toxin detection. The outbreak strain was characterized as an extended-spectrum β-lactamase- (ESBL-) expressing *E. coli* of serotype O104:H4 with virulence factors of both enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAggEC) [3]. Starting from 23rd of May stool culture for the outbreak strain was performed by screening for ESBL expressing *E. coli* (confirmed by VITEK 2 and *E*-Test, bioMérieux, Marcy l'Etoile, France) on culture media selective for STEC-serotypes attaining a high sensitivity. This was combined with testing for the presence of a Shiga toxin encoding phage confirmed by phenotypic Shiga toxin expression (as detected by RIDASCREEN Verotoxin ELISA, R-Biopharm AG, Darmstadt, Germany) effectuating high specificity. Additionally, in patients with proven STEC-infection serotype O104:H4 was confirmed by PCR as suggested by the Robert Koch Institute [4]. Using this diagnostic strategy, STEC O104:H4 infection was confirmed or excluded with very high sensitivity and specificity.

Five patients who had presented with respective symptoms between May 15th and 20th were contacted by hospital staff at the earliest possibility in order to obtain fecal testing and to establish follow-up for the rule-out of HUS-development. The survey was continued until July 2011, the time when German health authorities officially declared that the outbreak time period had stopped.

According to our ED standard-operating procedure, vital signs and body temperature were measured in almost every outbreak-related presenter. Moreover abdominal tenderness and bowel sounds were examined clinically and routine laboratory testing including HUS-indicating parameters was performed. The decision for hospital admission versus outpatient management was made according to the ED physician's clinical judgment. Outpatients who did not provide fecal specimen during their ED visit received a prepaid small package to send a stool sample at earliest possibility. All outbreak-related ED presenters who were not admitted received written instruction to establish clinical and laboratory follow-up at their general practitioner to rule out HUS development.

At the beginning of August 2011, all outbreak-related ED-presenters were contacted by postal mail and asked to fill in a supplemental questionnaire which served for cross-checking and/or completing anamnestic and anthropometric data. The survey and questionnaire were approved by the local ethics committee as "ad hoc" decision on 25th of May.

3. Statistics

Patients were retrospectively divided into two cohorts, STEC-positive and STEC-negative, according to their fecal microbiology result. STEC-negative patients were subdivided into those with fecal results positive for common enteropathogenic bacteria (other than STEC) or viruses and those with completely negative stool specimen.

We characterized these cohorts for anamnestic, clinical, and laboratory findings at their first ED contact and compared STEC-positive and STEC-negative (total and subgroups: "without stool pathogens" and "other stool pathogens") patients using Student's t-tests and χ^2-tests. Notably, due to the ESBL-selective testing mentioned above the sensitivity for the detection of other bacterial pathogens was limited, and, therefore, the definition of the subgroups of STEC-negative patients may be not completely reliable. A multiple logistic regression analysis was conducted to estimate odds ratios for positive versus negative STEC-infection. Anthropometric, anamnestic, clinical, and laboratory parameters were selected based on univariate testing and on clinical knowledge and were entered into the regression model.

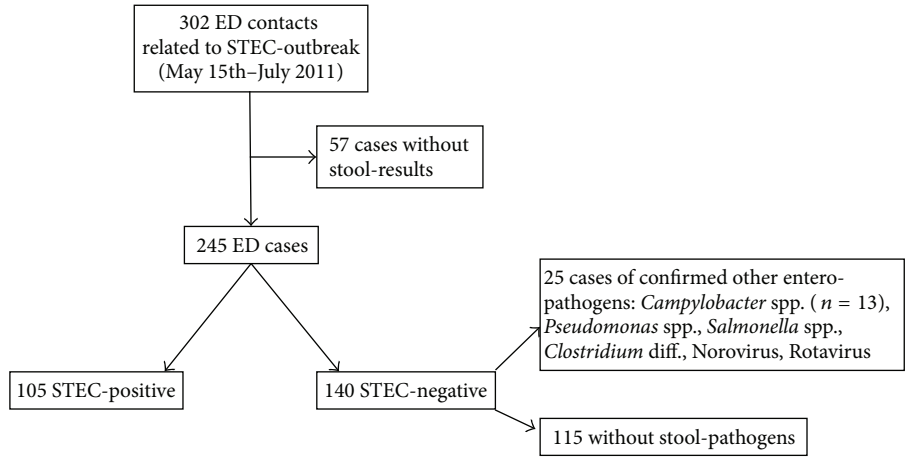

Figure 2: Enrollment of outbreak-related ED patients into the analysis.

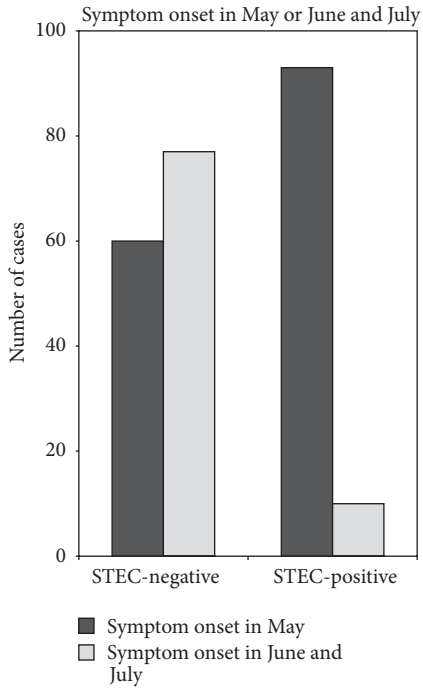

Figure 3: Month of onset of diarrheal symptoms in STEC-positive and STEC-negative ED contacts.

4. Results

During the STEC O104:H4 outbreak period, 302 adult patients with diarrheal complaint presented at the ED of any one of two tertiary hospitals in Luebeck. Fecal specimen was obtained in 245 (81%) patients: 105 were STEC-positive and 140 were STEC-negative; 25 patients of the STEC-negative cohort were positively tested for common enteropathogens (other than STEC). 57 (19%) did not return stool samples and were excluded from further analysis (Figure 2).

As shown in Table 1 anthropometric characteristics did not differ between both cohorts. Anamnestically, STEC-positive patients had a higher number of stools within last 24 h and had stronger complaint of lower abdominal pain. Visible bloody diarrhea was reported by 86% of STEC-positive compared to 59% of STEC-negative patients. Vital signs including body temperature, however, did not differ. At clinical examination 66% of STEC-positive versus 41% of STEC-negative patients had tenderness on abdominal palpation. At laboratory analysis the STEC-positive cohort showed slightly higher neutrophil counts and higher LDH, serum-creatinine, and bilirubin levels. These laboratory differences were consistent even if those cases were excluded who were already diagnosed for manifest HUS at their initial ED contact ($n = 17$).

However, the most striking difference between both groups was the time of symptom onset: in >90% of STEC-positive patients diarrheal symptoms had started in May 2011, whereas about 55% of STEC-negative presenters reported that diarrheal symptoms had started in June (Figure 3). As depicted in Figure 1(a) the daily numbers of ED contacts showed two overlapping clusters: the first occurred during the second half of May and was dominated by STEC-positive patients; the second phase occurred during the first half of June and was characterized by STEC-negative presenters.

5. Logistic Regression Analysis

Data consistency of most parameters was >80–90% except for BMI and defecation rate. However, due to missing items the multivariate logistic regression analysis was limited to 143 of 245 patients. We found that visible bloody diarrhea independently increased the risk of STEC-disease about 16-fold. The clinical finding of lower abdominal tenderness increased the risk about 5-fold. A defecation rate > 10/24 h did not attain statistical significance due to those 25 STEC-negative patients who were positive for other enteropathogens. Elevated blood-leukocyte counts were associated with an increased risk of STEC-infection. However, the parameter that was most significantly linked to STEC-positivity was the time period of diarrhea onset: the beginning of gastroenterocolitic symptoms in May 2011 increased the risk of STEC-infection about 40-fold (Table 2).

Table 1: Anamnestic, clinical, and laboratory findings in STEC-positive versus STEC-negative ED patients. STEC-negative cohort was subdivided into patients without microbiological finding of fecal pathogens and those having enteropathogens other than STEC.

	STEC-positive ($n = 105$)	STEC-negative					
		Total ($n = 140$)	p	Without stool pathogen ($n = 115$)	p	Other stool pathogens ($n = 25$)	p
Anthropometric and anamnestic parameter							
Age (years, mean ± SD)	52.4 ± 20.2	43.1 ± 19.1	**<0.001**	43.5 ± 19.3	**0.001**	41.3 ± 18.4	0.614
BMI (kg/m^2, mean ± SD)	25.5 ± 5.5	25.5 ± 4.5	0.976	25.0 ± 4.4	0.531	27.6 ± 4.8	**0.028**
Female (% of patients)	57.1	63.6	0.308	64.3	0.274	60.0	0.682
Smoker (% of patients)	23.0	32.5	0.140	33.7	0.113	27.3	0.563
Start of symptoms (% of patients)							
May 2011	90.3	43.2	**<0.001**	41.7	**<0.001**	50.0	0.643
June/July 2011	9.7	55.4		56.2		50.0	
Defecation rate > 10/d (% of pat.)	56.9	23.2	**<0.001**	17.6	**<0.001**	52.9	**0.005**
Faeces "bloody" (% of patients)	86.4	58.7	**<0.001**	63.2	**<0.001**	37.5	**0.020**
Nausea (% of patients)	50.0	55.0	0.451	51.4	0.839	70.8	0.085
Vomiting (% of patients)	42.2	34.6	0.232	28.6	**0.037**	62.5	**0.002**
Abdominal pain (% of patients)	92.0	83.6	0.057	80.9	**0.020**	95.8	0.074
Pain at lower abdomen (% of patients)	71.0	41.0	**<0.001**	40.1	**<0.001**	41.7	0.283
Fever (>38°C; % of patients)	4.0	9.4	0.113	7.0	0.347	20.8	**0.035**
Clinical findings							
Body temperature (°C, mean ± SD)	37.1 ± 0.5	37.1 ± 0.7	0.682	37.0 ± 0.7	0.210	37.4 ± 0.7	**0.012**
Blood pressure (mmHg, mean ± SD)							
Systolic	133 ± 20	131 ± 22	0.468	132 ± 23	0.617	129 ± 20	0.554
Diastolic	78 ± 11	76 ± 11	0.141	77 ± 12	0.318	74 ± 9	0.202
Heart rate (min^{-1}, mean ± SD)	80.4 ± 16.2	81.1 ± 15.8	0.758	79.9 ± 15.7	0.824	87.5 ± 15.2	0.060
Abdominal tenderness (% of patients)	65.6	40.9	**<0.001**	39.1	**<0.001**	50.0	0.342
Tenderness lower abdomen (% of patients)	56.3	26.2	**<0.001**	21.7	**<0.001**	36.4	0.651
Bowel sounds present (% of patients)	96.9	98.4	0.474	90.4	0.169	90.9	**0.003**
Signs of dehydration (% of patients)	31.9	18.0	**0.016**	12.3	**0.001**	45.5	**<0.001**
Laboratory findings							
Total leukocytes (/μL, mean ± SD)	10725 ± 4286	9467 ± 3737	**0.019**	9230 ± 3712	**0.009**	10431 ± 3754	0.151
Creatinine (μmol/L, mean ± SD)	116.7 ± 150.1	71.3 ± 18.7	**0.001**	70.7 ± 16.5	**0.002**	73.8 ± 26.2	0.464
LDH (U/L, mean ± SD)	270.3 ± 334.2	172.3 ± 42.4	**0.001**	170.5 ± 41.7	**0.003**	179.5 ± 45.6	0.354
Total bilirubin (μmol/L, mean ± SD)	18.2 ± 25.7	10.4 ± 7.7	**0.002**	9.4 ± 5.1	**0.001**	14.9 ± 13.4	**0.002**
CRP (mg/L, mean ± SD)	25.6 ± 33.4	27.3 ± 52.7	0.775	15.4 ± 32.2	**0.032**	74.8 ± 84.4	**<0.001**
Thrombocytes (/nL, mean ± SD)	207 ± 79.0	221 ± 65.8	0.130	227 ± 53.5	**0.040**	197 ± 64.3	**0.018**
Hemoglobin (g/L, mean ± SD)	135 ± 24.9	137 ± 19.8	0.446	136 ± 19.9	0.686	141 ± 19.2	0.260
Hematocrit (/L/L, mean ± SD)	0.40 ± 0.1	0.41 ± 0.1	0.352	0.40 ± 0.0	0.567	0.42 ± 0.1	0.233

Table 2: Multivariate logistic regression analysis for STEC-positive versus STEC-negative ED contacts. Odds ratio indicates the respective risk of being STEC-infected.

	Odds Ratio	95% Confidence interval	p value
Start of symptoms (May versus June/July)	**40.885**	**(7.870–212.393)**	**<0.001**
Defecation rate (>10 versus <10/24 h)	1,993	(0.545–7.279)	0.297
Bloody diarrhea	**15.056**	**(2.014–112.558)**	**0.008**
Anamnestic lower abdominal pain	2.969	(0.824–10.673)	0.096
Lower abdominal tenderness	**4.637**	**(1.186–18.124)**	**0.027**
Elevated C-reactive protein	0.592	(0.137–2.562)	0.483
LDH (U/L)	1.011	(0.999–1.024)	0.082
Bilirubin (μmol/L)	1.064	(0.981–1.155)	0.133
Creatinine (μmol/L)	1.033	(1.001–1.065)	0.044
Elevated leukocyte count	**16.617**	**(2.316–119.243)**	**0.005**

6. Discussion

The main finding of the present observational study is that the ED management of the German STEC O104:H4 outbreak involved not only STEC-positive patients, but at even greater quantity STEC-negative ED contacts. This phenomenon of "STEC + X" is largely ignored by epidemiologic statistics. As depicted in Figure 1(a) the daily numbers of outbreak-related ED contacts were not uniformly distributed. Roughly two main overlapping clusters could be distinguished: The first peaked during the second half of May with the majority of patients tested positively for STEC-infection. This early cluster closely corresponds to the Gaussian distribution of the nationwide epidemiology curve that was presented by the RKI in September 2011 (Figure 1(b)) [1]. Slight differences between the nationwide curve (Figure 1(b)) with peak on May 22nd and our local data (Figure 1(a)) with peak on May 26th might result from the fact that date of ED contact (local Figure 1(a)) includes some delay from start of diarrheal symptoms (nationwide Figure 1(b)).

The second cluster of ED presentations peaked at about 4th of June. This second wave of contacts is dominated by STEC-negative subjects. Interestingly, the days of highest contact rates do well coincide with alarming regional news in public media: on May 28th STEC-related deaths including one old patient in Luebeck were reported [5]; on June 4th a well-known local restaurant was identified for dissemination of STEC-contaminated food [6]. The seriousness of the illness and the fatalities, coupled with the lack of a definitive source of the causative agent, created uneasiness among the public. Obviously, the local involvement into the STEC-tragedy further boosted ED contact rates.

The phenomenon that the media coverage might significantly increase public uneasiness with alarming reports is well-known from the H1N1-pandemics [7]. While it is the business of public media to sell news, wild headlines are not helpful to gaining public trust and cooperation with health agencies in controlling the spread of an outbreak. Moreover, during the German STEC-O104:H4 outbreak, a close epidemiologic surveillance was significantly delayed due to bureaucratic decentralized reporting pathways involving local, federal state, and national levels of health authorities [8]. The mean reporting delay was estimated 1-2 weeks [1]. Given the fact that the outbreak strain showed a prolonged median incubation period of 8 days up to the onset of diarrheal symptoms, as compared to experience from previous outbreak investigations with EHEC O157 (3 to 4 days), public and official awareness of the epidemic was congruent with the second peak of ED contacts (mainly STEC-negative) but not with the real peak of the infection interval (Figure 1(b)).

Since bloody diarrhea is frequently the first symptom that EHEC patients experience, the development of an EHEC outbreak can be assessed almost real-time by ascertaining patients presenting with these symptoms, for example, in emergency departments [9]. Therefore, on May 27, 2011, syndromic surveillance of patients with bloody diarrhea was established in collaboration of emergency departments and the RKI [8]. This still was subject to some bias but effectively helped to correct the official estimate of the outbreak during June 2011 [1].

At initial ED contact a definite distinction of STEC-positive and STEC-negative patients by clinical judgment alone was not possible and clinical criteria for the risk of future HUS-development were unknown. In the light of our early clinical experience since May 15th that even oligosymptomatic patients with only mild STEC-gastroenteritis might deteriorate to severe HUS within few days, stratification for the need of follow-up was not possible until microbiological results of the respective fecal testing were available. According to the "Practice Guidelines for the Management of Infectious Diarrhea" and recent recommendations [10, 11] gastroenteritic symptoms with passage of small-volume stool containing visible blood as well as a suspected outbreak should prompt fecal culture of enteropathogenic bacteria including STEC. Therefore, stool sampling was rigorously initiated either during the ED or hospital stay or via packages with prepared sampling kits. Though some of these kits were not returned, we have valid STEC-results in >80% of ED presenters due to the very high sensitivity and specificity of the combined approach using ESBL-culture, ELISA, and PCR-tools.

The univariate and multivariate comparison of the outbreak-related STEC-positive versus STEC-negative ED cohort revealed that the visible presence of blood in the

feces as well as lower abdominal tenderness was clearly associated to STEC-positivity. This is in accordance with previous reports [10]. A high defecation rate (>10/24 h) did not distinguish between STEC-infected cases and those patients who had positive fecal testing for enteropathogens other than STEC. This heterogeneous group of 25 patients, comprising 13 subjects infected with *Campylobacter* spp., was too small for an extended separate analysis. Though blood leukocyte counts, serum creatinine, and bilirubin showed statistic differences between both cohorts they did not reliably approve or exclude STEC-infection. Likewise other anamnestic, clinical, and laboratory parameters were not helpful for the discrimination between STEC-positive and STEC-negative cohorts. In contrast, the time of symptom onset was by far the strongest risk factor for STEC-infection. In our retrospective analysis >90% of STEC-positive patients developed diarrhea in May 2011.

7. Strength and Limitations

Inherent to the unpredictable nature of outbreaks this is an unplanned observational study. Hence, in our cohorts data are not complete for every patient who met the inclusion criteria. However, data consistency was >80–90% for all parameters except for BMI and defecation rate. Standardization of our anamnestic, clinical, and laboratory diagnostic and management early in the course of the outbreak provided sufficient statistical power. We did not aim at calculating a combined clinical risk score to estimate high probability versus exclusion of STEC-infection, because this approach appears to be of minor utility with regard to the overwhelming outbreak-related temporal frame.

8. Conclusion and Perspective

During the STEC O104:H4 outbreak in 2011 EDs had to manage STEC-positive as well as STEC-negative diarrhea patients. Though some anamnestic, clinical, and laboratory findings were significantly different between STEC-positive and STEC-negative cohorts, these parameters seem not suitable to reliably discriminate between both patient groups. The risk of STEC-positivity was tightly linked to the period of symptom onset corresponding to the peak of new infections in retrospective nationwide epidemiologic reports.

EDs offer contact to patients 24 h/7 days. They are strongly involved in the management of any infectious epidemic. Close collaboration with the microbiology department is mandatory for efficient surveillance. Bloody diarrhea is a well-established trigger of fecal diagnostic [9–11]. Subtype-specific microbiologic work-up for foodborne infections like STEC can identify whether sudden increases in reported cases are due to sporadic cases or to one or more outbreaks [12]. The costs and potential benefits of subtype-specific surveillance tools have been discussed elsewhere previously [13, 14]. Given the possibility of long-term-shedding in a high proportion of infected individuals, as found in the O104:H4 epidemic, such surveillance would be reasonable even beyond the mere outbreak period [15]. EDs are the optimal partners for central health authorities: close communication via direct reporting systems can avoid reporting delays and seismographically survey the outbreak. Good public communication is essential, but communication failures delay outbreak control, undermine public trust, and unnecessarily prolong economic, social, and political turmoil.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Johannes Knobloch and Martin Nitschke contributed equally.

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Research Article

UV-Heat Treatments for the Control of Foodborne Microbial Pathogens in Chicken Broth

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This investigation established the process criteria for using UV-C light and mild heat (UV-H treatment) to inactivate 5-Log_{10} cycles (performance criterion) of common foodborne pathogen populations, *Escherichia coli*, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus*, when inoculated in chicken broth. To define the target microorganism and the proper UV-H treatment conditions (including UV dose, treatment time, and temperature) that would achieve the stated performance criterion, mathematical equations based on Geeraerd's model were developed for each microorganism. For the sake of comparison, inactivation equations for heat treatments were also performed on the same chicken broth and for the same microorganisms. *L. monocytogenes* was the most UV-H resistant microorganism at all temperatures, requiring a UV dose between 6.10 J/mL (5.6 min) and 2.26 J/mL (2.09 min) to achieve 5-Log_{10} reductions. In comparison with UV treatments at room temperatures, the combination of UV and mild heat allowed both the UV dose and treatment time to be reduced by 30% and 63% at 55°C and 60°C, respectively. Compared to heat treatments, the UV-H process reduced the heating time for 5-Log_{10} reductions of all the investigated microorganisms in chicken broth from 20-fold to 2-fold when the operating temperature varied from 53 to 60°C.

1. Introduction

Despite the fact that conventional heat treatments still prevail in the food industry, several nonthermal food processing technologies have emerged recently. Compelled by the strong preference of consumers for fresh and minimally processed food, research into food processing has focused on the investigation of alternatives to pasteurization, which has long been popular for its ability to ensure microbial inactivation at lower temperatures while minimizing losses of the organoleptic and nutritional properties of food. One of these technologies is short wave ultraviolet radiation (UV-C), which has numerous advantages, including the ability to inactivate a wide range of pathogenic and spoilage microorganisms in juices [1, 2], thereby minimizing the loss of nutritional and sensorial quality [3]. Moreover, UV-C does not generate chemical residues or toxic compounds [4] and requires very little energy consumption compared with other nonthermal pasteurization processes [5]. The germicidal properties of UV-C light rely on DNA's absorption of the UV light, which induces structural distortions in the DNA molecule, inhibiting transcription and replication and eventually leading to cell death.

Although previous studies have shown that UV-C radiation can be an effective method to inactivate microorganisms in liquid food, practical applications of this technology are limited due to its low penetration capacity in liquids with high absorption coefficient and turbidity. It is well known that the optical properties of the treatment medium strongly influence the lethal effect of UV light [6] due to absorption, reflection, scattering, and refraction phenomena caused by the presence of color compounds and soluble or suspended particles. In order to overcome these limitations, combinations of UV radiation with other nonthermal technologies, such as pulsed electric fields, have been designed, based on hurdles technology approach. For instance, it has been demonstrated that UV light followed by pulsed electric fields (PEF) treatments has an additive effect [7].

Recently, we have demonstrated that the simultaneous application of UV radiation and heat at sublethal temperatures (UV-H treatment) remarkably enhances the former's microbial inactivation capacity. Specifically, studies on the inactivation of various bacterial species, both in buffers and fruit juices (orange and apple), have shown that UV-H treatments result in a synergistic lethal effect, with the temperature of maximum synergism being different for each bacterial species [8–10]. However, so far, our studies have focused on low pH media, where acidity can reduce the heat resistance of bacteria and, consequently, enhance the effectiveness of UV-H treatments. Additionally, data on food with more neutral pH are scarce but do include some studies on egg white [11–13]. Therefore, the question of whether a similar synergistic lethal effect could also occur in other liquid foods with different pH, chemical composition, and absorption coefficients was raised.

To challenge the inactivation effect of UV-H treatments on complex food matrices, chicken broth was chosen as a multi-ingredient liquid food with higher pH and a more complex chemical composition that provides a favorable environment for bacteria. The goal of this study was to investigate whether the UV-H combined process can be applied to liquid food that has a high absorption coefficient, turbidity, and low acidity such as chicken broth, resulting in a synergistic lethal effect comparable with/similar to that which has been obtained in the case of fruit juices. For this study, we have used the most UV resistant strains of different pathogenic bacteria as reference microorganisms, such as *Escherichia coli*, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Staphylococcus aureus*.

2. Materials and Methods

2.1. Bacterial Culture and Media. The strains of *E. coli* STCC 4201, *Salmonella* Typhimurium STCC 878, *L. monocytogenes* STCC 5672, and *S. aureus* STCC 4465 were obtained from the Spanish Type Culture Collection (STCC). The bacterial cultures were maintained frozen at –80°C in cryovials. A broth subculture was prepared by inoculating 10 mL of tryptone soy broth (Biolife, Milan, Italy) supplemented with 0.6% (w/v) yeast extract (Biolife) (TSBYE) with a loopful of growth from tryptone soy agar (Biolife) supplemented with 0.6% (w/v) yeast extract (TSAYE). The subculture was incubated at 35°C for 6–12 h in a shaking incubator (150 rpm; Heidolph Instruments, Vibramax 100, Schwabach, Germany). With these subcultures, 250 mL flasks containing 50 mL of TSBYE were inoculated to reach a concentration of 10^4 CFU/mL and then incubated for 24 h under the same conditions until the stationary growth phase was reached (2×10^9 CFU/mL).

2.2. UV Equipment and Treatments. UV treatments were carried out in a unit with 8 individual annular thin film flow-through reactors connected in a series and equipped with a feed tank and a peristaltic pump (ISM 10785, Ismatec, Glattbrugg, Switzerland), as described previously by Gayán et al. [8]. Each reactor included a low-pressure mercury vapor lamp (8 W of input power; model TUV 8WT5, Philips, USA), which converted 30% of the input power as UV-C radiation (Philips Electronics, 2012), emitting 85% of UV-C energy at 254 nm. The lamp was attached to the axis of an outer glass tube (25 mm of inner diameter) and was enclosed using a quartz tube (20 mm of outer diameter) to prevent direct contact of the lamp with the treatment medium. In the annular gap (2.5 mm), a stainless steel coil spring was installed to improve the flow's turbulence. The outside and inside coil diameters of the spring were 23 mm and 25 mm, respectively, and its length and pitch were 270 mm and 10 mm, respectively. A manual sampling valve was located in the outlet of each reactor. The entire unit was submerged in a 90 L water bath (25.0–60.0°C) heated by the circulating water of a peripheral thermostatic bath (Kattebad K12, Huber, Offenburg, Germany). The equipment also included a heating/cooling coil exchanger at the inlet of the first reactor. Thermocouples (ZA 020-FS, Almeco, Bernburg, Germany) fitted to the inlet and outlet of the first and last reactor, respectively, allowed for treatment temperature control.

Chicken broth (absorption coefficient = 19.6 cm^{-1}, turbidity = 4460 NTU, pH = 5.2) for use as treatment medium was purchased from a local market (Interal S.A., Spain). Broth's absorption coefficient was measured spectrophotometrically (254 nm; UV500, Unicam Limited, Cambridge, UK). Samples were diluted and evaluated using quartz cuvettes (Hellma, Müllheim, Germany) with path lengths of 1 mm, 2 mm, and 10 mm. The absorption coefficient of the diluted samples was determined from the slope of the absorbance versus the path length and corrected by the dilution factor. Turbidity was measured with a nephelometer (HI 83749, Hanna Instrument, Szeged, Hungary). pH was measured using a pH-meter Basic 20 (Crison Instrument, Barcelona, Spain). The chicken broth was inoculated with the bacterial suspension to achieve 10^5–10^7 CFU/mL and pumped (8.5 L/h) through the heat exchanger to the reactors. When the treatment conditions were stabilized, samples were withdrawn through the sampling valves and 0.1 mL or 1 mL was immediately pour-plated into the recovery medium.

2.3. Heat Treatments. Heat treatments were carried out in specially designed thermoresistometer TR-SC [14]. This instrument consisted of a 400 mL vessel with an electrical heater for thermostation, an agitation device used to ensure inoculum distribution and temperature homogeneity, a pressurization system, and ports for injecting the microbial suspension and for extraction of samples. Once the preset temperature had attained stability ($T \pm 0.05$°C), 0.2 mL of an adequately diluted microbial cell suspension was inoculated into the vessel, which contained the 350 mL of chicken broth. After inoculation, 0.2 mL samples were collected at different heating times and were immediately pour-plated.

2.4. Incubation of Treated Samples and Survival Counting. TSAYE was used as a recovery medium and the plates were incubated at 35°C for 24 h for *E. coli*, *Salmonella* Typhimurium, and *S. aureus* and for 48 h for *L. monocytogenes*. After incubation, colony forming units (CFU) were counted using an improved Image Analyzer Automatic Colony Counter

(Protos, Synoptics, Cambridge, UK), as described elsewhere [15].

2.5. Curve Fitting and Dose Calculation. Survival curves were obtained by plotting the logarithm of the survival fraction *versus* UV dose (d) expressed in joules per milliliter and time (t) expressed in minutes for UV and heat treatments, respectively. To compare UV-H treatments with thermal treatments, UV-H survival curves were also expressed in treatment time. The UV dose delivered to the treatment medium was estimated with a chemical dosimeter. To this end, the iodide-iodate actinometer (quantum yield = 0.73 ± 0.02) was used, following the indications of Rahn et al. [16]. The actinometer buffer was pumped through the installation at 8.5 L/h and the increase in absorbance (352 nm) was determined at the outlet of each reactor [8]. From this data, the photon flux (254 nm), as received per second by each volume fraction of the treatment medium, was estimated according to Montalti et al. [17]. Thus, considering the energy of a photon at 254 nm, the UV dose delivered to each reactor was 0.49 J/mL.

To fit the survival curves obtained at each temperature and to calculate resistance parameters, the GInaFiT inactivation model-fitting tool was used [18]. Specifically, the log-linear regression plus shoulder model from Geeraerd et al. [19] was chosen (1) since most of the survival curves exhibited shoulders. This model describes the survival curves through two parameters: the shoulder length (Sl), defined as dose or time before the exponential inactivation begins, and the inactivation rate ($K_{\max}$), defined as the slope of the exponential part of the survival curve. N_0 and N_t represent the initial numbers of the microbial population and the number of microorganisms that survive at the end of the treatment time (t), respectively. Consider the following equation:

$$N_t = N_0 e^{-K_{\max}\mathrm{Sl}} \left(\frac{e^{K_{\max}\mathrm{Sl}}}{1 + \left(e^{K_{\max}\mathrm{Sl}} - 1\right) e^{K_{\max} t}} \right). \quad (1)$$

To describe the relationship between treatment temperature (T) and Sl and $K_{\max}$ parameters, mathematical equations based on the Weibull distribution were chosen. For Sl, the equation introduced by Albert and Mafart [20] (2) was used as a secondary model, whereas the thermodependence of $K_{\max}$ was described using the mirror image of the Mafart [21] model (3):

$$\mathrm{Sl}_T = \left(\mathrm{Sl}_0 - \mathrm{Sl}_{\mathrm{res}}\right) 10^{-(t/\delta)^p} + \mathrm{Sl}_{\mathrm{res}}, \quad (2)$$

$$K_{\max T} = \left\lfloor K_{\max 0} 10^{-(t/\delta)^p} \right\rfloor, \quad (3)$$

where Sl_T and $K_{\max T}$ are the shoulder length and the inactivation rate of UV-H treatments at temperature T, respectively; Sl_0 and $K_{\max 0}$ are the shoulder length and the inactivation rate of the survival curves of UV treatments at room temperature, respectively; and $\mathrm{Sl}_{\mathrm{res}}$ is the residual shoulder when the treatment temperature was increased. δ and p are, respectively, the scale and shape parameters. The δ value represents the temperature increase necessary to achieve the first decimal reduction of Sl or $K_{\max}$ (from Sl_0 and $K_{\max 0}$ to $\mathrm{Sl}_0/10$ and $K_{\max 0}/10$). The p parameter ($p > 1$) accounts for the profile of the downward concavity of curves [20, 21]

For the heat survival curves, which showed an initial shoulder phase, the Geeraerd model was also used as a primary model. In order to study the relationship between the inactivation model parameters and the treatment temperature, Albert and Mafart's equation [20] was used for Sl, as described above, and simple log-linear equations were used for $K_{\max}$, while the slope and the intercept of the regression line were considered model parameters. The coefficient of determination (R^2), the root mean square error (RMSE), the bias (B_f), and accuracy (A_f) factors were used to determine the goodness of fits of both the primary and the secondary models, as well as the accuracy of the final equations [22]. The bias factor indicates systematic over-($B_f > 1$) or under-($B_f < 1$) prediction of the observed data. On the other hand, the accuracy factor indicates the extent to which the predictions differ from the observed data.

2.6. Statistical Analyses. Statistical analyses, a t-test, and an ANOVA test were carried out using the GraphPad PRISM 5.0 software (GraphPad Inc., San Diego, CA, USA) and differences were considered significant for $P \leq 0.05$. All microbial resistance determinations, as well as analytical assays, were performed at least three times on different workings days. The error bars in the figures correspond to the mean standard deviation.

3. Results and Discussion

This study investigated the thermodependence of the UV inactivation of pathogenic bacteria in chicken broth. The intention was to establish the UV-H treatment conditions (process criteria) necessary for obtaining a certain level of inactivation of the pathogenic microorganisms of reference. For this purpose, the effect of temperature on the UV lethality of UV tolerant strains of *E. coli* (STCC 4201), *Salmonella* Typhimurium (STCC 878), *L. monocytogenes* (STCC 5672), and *S. aureus* (STCC 4465) was assessed. The strains used in this investigation were the most UV resistant among five different strains of each investigated microorganism according to previous studies [8, 23–25] carried out under the same methodology. In particular, the selected strains of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* were 15.6, 15.1, 15.8, and 2.7% more resistant than the second most resistant strain of each microorganism.

To describe this influence and to define the process criteria, mathematical equations, including UV dose, time, and temperature, have been developed. In order to compare results, the heat resistance of the indicated microorganisms was also investigated and modeled.

3.1. Microbial Inactivation by UV-H Treatments in Chicken Broth. The survival curves of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* to UV treatments at room temperature and at 50.0, 52.5, 55.0, 57.5, and 60.0°C (UV-H) are presented in Figure 1. An initial lag phase

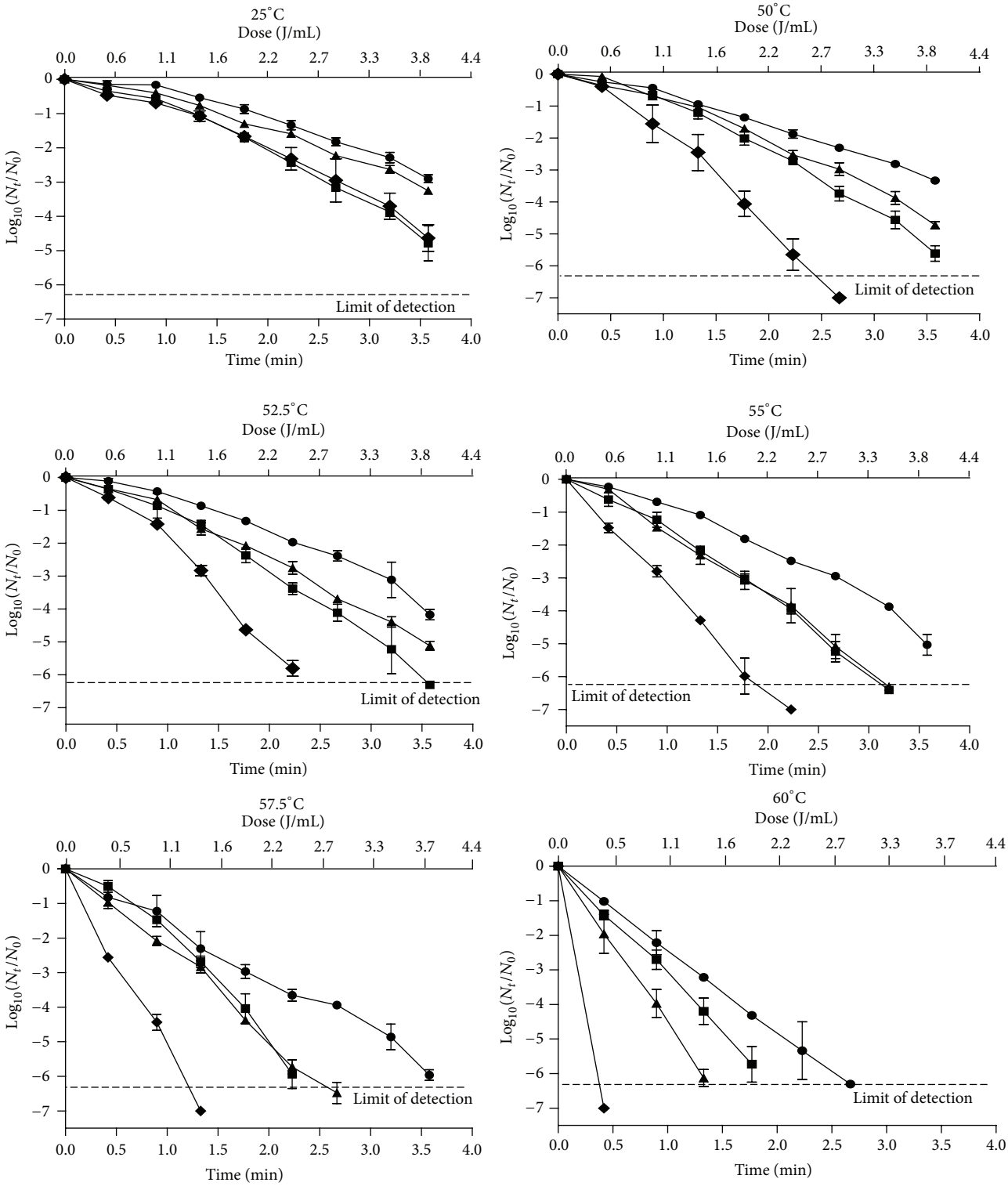

Figure 1: Survival curves of *E. coli* (STCC 4201) (■), *Salmonella* Typhimurium (STCC 878) (▲), *L. monocytogenes* (STCC 5672) (●), and *S. aureus* (STCC 4465) (◆) to UV treatment at room temperature (25°C) and to combined UV-H treatments at 50.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C in chicken broth.

(shoulder) was observed for most of the survival curves. After this, the microbial death followed a logarithmic order, but no tailing took place. UV-H inactivation curves displayed shoulder phases, which are often observed in survival curves for UV-C light [26, 27]. According to the "multihit target theory," shoulders are related to the DNA damage and repair phenomena [28]. DNA repair systems can repair damage up to certain UV doses, resulting in shoulders. Once the maximum DNA repair capability is surpassed, additional UV exposure is lethal for microorganisms and survivors exponentially decline [29].

When the maximum UV dose possible in one pass through the equipment (3.92 J/mL) was applied, UV treatments at room temperature (25°C) decreased the microbial population of the investigated bacteria from 5-Log_{10} cycles for *E. coli* and *S. aureus* to 3-Log_{10} cycles for *Salmonella* Typhimurium and *L. monocytogenes*, which showed the highest UV resistance (Figure 1). This level of inactivation for the same bacteria was higher than that observed in other products like fruit juices that have lower turbidity but higher absorption coefficient. Thus, Gouma et al. [30] observed hardly 1-Log_{10} reductions of these microorganisms in apple juice (turbidity 7.4 NTU, $\alpha = 24\ cm^{-1}$) after applying the same UV treatment at 25°C in the same facility. Since pH did not affect the UV lethality [23] and since the turbidity of the chicken broth (4460 NTU) was higher than that of the apple juice, the larger inactivation observed in this investigation could be due to the slightly lower absorption coefficient of the chicken broth ($19.6\ cm^{-1}$) compared to that of the apple juice. This lower absorption coefficient would cause less UV-C light to be absorbed by the treatment medium, meaning that the same UV dose would have more bactericidal effect in the chicken broth than in the apple juice [8]. Also, any component of the chicken broth could interact on the microbial UV resistance, thereby increasing the UV lethality.

Although 3-Log_{10} cycles of inactivation were achieved after the maximum applied UV dose at 25°C for all the investigated pathogenic microorganisms, which were selected in previous studies due to their being the most UV resistant [8, 23–25], a reduction of 99.9% of the microbial population could not be sufficient to ensure the safety of chicken broth; for example, at least 5-Log_{10} reductions of the pathogen of reference are necessary for the pasteurization of fruit juices [31]. Therefore, it would be necessary to increase the lethality of UV treatments.

The application of UV at moderate temperatures has resulted in a synergistic increase of UV lethality [11, 13]. In this investigation, when the treatment temperature was raised between 50.0°C and 60.0°C (Figure 1), the UV inactivation of all investigated microorganisms improved considerably. For instance, the UV inactivation of the most resistant microorganism, *L. monocytogenes*, with a dose of 2.45 J/mL (2.23 min), increased from 1.34-Log_{10} cycles at 25°C to 1.87, 1.96, 2.48, 3.66, and 5.35-Log_{10} cycles at 50.0°C, 52.5°C, 55.0°C, 57.5°C, and 60.0°C, respectively. These results indicated that combining UV light with mild heat increased the UV inactivation of microorganisms in chicken broth, which paralleled the observation of a higher effect of UV and heat in other food products. This fact suggests the possibility of designing a feasible UV-H hygienization process for this kind of product (i.e., broth), which is, apparently, difficult to treat with this technology due to its high turbidity and absorption coefficient.

Although an increment of the UV lethality has been observed when augmenting the temperature, the different behavior of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* in response to UV-H treatments in relation to the treatment temperature makes it difficult to compare data. Moreover, the obtained survival curves showed shoulders at lower temperatures that disappeared when the temperature was increased, which limited the application of simpler first order inactivation kinetics. Therefore, it was necessary to develop mathematical models that enabled an evaluation of the effect of temperature on UV lethality in chicken broth for each investigated microorganism.

To describe UV-H inactivation kinetics, the log-linear regression plus the shoulder model of Geeraerd et al. [19] (1) (primary model) was used because it allowed the length of the shoulders and the log-linear rate of inactivation to be accurately and independently described. Table 1 includes the averages and the standard deviations of the model parameters (K_{max} and Sl), expressed in time terms, obtained from the fitting of the UV-H survival curves of all microorganisms tested at different temperatures. The coefficient of determination (R^2) and the root mean square error (RMSE) values are also included to illustrate the goodness of the fits. As observed, the UV lethality improved when the treatment temperature was raised and stemmed from the decrease of the shoulder phase (Sl) of the UV-H survival curves until it disappeared. In addition, the slope of the survival curves (K_{max}) increased with the rising temperature. Furthermore, the UV resistance variability between species was maintained at different temperatures and was reflected in the Sl and K_{max} values. Thus, the higher resistance of *L. monocytogenes* was due to larger values of Sl and smaller values of K_{max}. On the contrary, *S. aureus* was the most sensitive microorganism, showing smaller Sl values and higher K_{max} ones. Finally, the Gram negative bacteria *E. coli* and *Salmonella* Typhimurium showed a similar behavior and Sl and K_{max} values. In general, it is believed that Gram positive bacteria are more UV resistant than Gram negative, which can be attributed to the thicker peptidoglycan cell wall of the former [32, 33]. In this study, the UV resistance of *S. aureus* at 25°C was similar to that of *E. coli* and slightly lower than that of *Salmonella* Typhimurium, which demonstrates that this statement should not be considered a general rule. In fact, other authors have reported a higher susceptibility of *S. aureus* to UV technologies compared with coliforms [34, 35]. At higher temperatures, that statement cannot be maintained since heat interferes on the microbial UV resistance. For example, the highest UV-H lethality for *S. aureus* would be due to the higher heat sensitivity of this microorganism compared with the other investigated bacteria as it will presented later on.

To describe the effect of the changes in temperature on the kinetic parameters obtained after fitting the primary model to the UV-H inactivation data of *E. coli*, *Salmonella* Typhimurium, *S. aureus*, and *L. monocytogenes* in chicken broth (shown in Table 1), the corresponding secondary models for

Table 1: Resistance parameters (Sl and K_{max}) obtained from the fit of UV-H survival curves of *E. coli*, *Salmonella* Typhimurium, *S. aureus*, and *L. monocytogenes* at different temperatures in chicken broth to Geeraerd's model (1). Estimated standard deviations (SD) of the means are in parentheses. Letters a, b, c, and d indicate statistically significant differences ($P \leq 0.05$) among Sl and K_{max} values of UV-H survival curves of different microorganisms at the same treatment temperature.

Microorganism	Temperature (°C)	Sl (min)	K_{max} (min^{-1})	RMSE	R^2
Escherichia coli	25.0	0.67 (0.09)[a]	3.66 (0.34)[a]	0.152	0.994
	50.0	0.68 (0.15)[a]	4.31 (0.22)[a]	0.185	0.993
	52.5	0.55 (0.16)[a]	4.59 (0.20)[a]	0.228	0.990
	55.0	0.33 (0.11)[a]	5.06 (0.34)[a]	0.229	0.991
	57.5	0.10 (0.06)[a]	6.00 (0.43)[a]	0.329	0.985
	60.0	0.00 (0.00)[a]	7.33 (0.62)[a]	0.153	0.996
Salmonella Typhimurium	25.0	0.68 (0.04)[a]	2.52 (0.01)[b]	0.098	0.995
	50.0	0.65 (0.10)[a]	3.58 (0.27)[b]	0.144	0.994
	52.5	0.45 (0.02)[a]	3.78 (0.04)[b]	0.140	0.996
	55.0	0.30 (0.10)[a]	4.91 (0.05)[a]	0.219	0.992
	57.5	0.09 (0.05)[a]	5.91 (0.27)[a]	0.261	0.991
	60.0	0.00 (0.00)[a]	10.47 (0.74)[b]	0.256	0.993
Listeria monocytogenes	25.0	1.08 (0.13)[b]	2.55 (0.02)[c]	0.077	0.996
	50.0	1.00 (0.02)[a]	2.70 (0.02)[c]	0.068	0.997
	52.5	0.89 (0.04)[b]	3.24 (0.32)[c]	0.212	0.988
	55.0	0.73 (0.13)[b]	3.68 (0.32)[b]	0.212	0.988
	57.5	0.20 (0.05)[b]	4.20 (0.03)[b]	0.308	0.977
	60.0	0.00 (0.00)[a]	5.60 (0.42)[c]	0.253	0.989
Staphylococcus aureus	25.0	0.59 (0.17)[a]	3.37 (0.35)[d]	0.204	0.987
	50.0	0.41 (0.09)[a]	5.41 (0.15)[d]	0.222	0.995
	52.5	0.38 (0.01)[a]	6.50 (0.10)[d]	0.279	0.991
	55.0	0.25 (0.02)[c]	7.71 (0.55)[c]	0.213	0.995
	57.5	0.10 (0.00)[a]	11.93 (0.24)[c]	0.3521	0.988

each microorganism were developed. Figure 2 depicts the relationship between temperature and the Sl and K_{max} values for all the investigated microorganisms. Mathematical equations (secondary models) based on the Weibull distribution—Albert and Mafart's equation (2) for Sl and Mafart's equation (3) for K_{max}—were used to describe the thermodependence of both parameters. Table 2 compiles the obtained parameters (δ, p, Sl_0, Sl_{res}, and $K_{max\,0}$) from the secondary models of Sl and K_{max} for each microorganism, including the R^2 and RMSE values from the fits. The relationship between the Sl (Figure 2(a)) and the temperature displayed a sigmoid profile for all microorganisms, first showing a lag phase and then dropping off to zero. *L. monocytogenes* showed higher shoulder length values of the UV-H survival curves than all the other species at all treatment temperatures tested, especially at temperatures ranging from 25°C to 55.0°C. Above this value, differences were reduced until the shoulder length became zero. When K_{max} values were plotted against treatment temperature, concave upward curves were observed (Figure 2(b)). The inactivation rate of *S. aureus* and *Salmonella* Typhimurium was more sensitive to temperature changes than that of *E. coli* and *L. monocytogenes*. This behavior was evidenced in the scale parameter (δ) of K_{max} secondary models, which determined the temperature increment that reduced the K_{max} 10-fold (Table 2). δ values for *S. aureus* and *Salmonella* Typhimurium were smaller than those obtained for *L. monocytogenes* and *E. coli*.

To describe, compare, and predict the microbial inactivation by UV-H treatments of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* in chicken broth, final equations were developed. These equations were obtained by introducing the secondary models for Sl and K_{max} values (2) and (3) into Geeraerd's primary model (1). To show the goodness of the fits of the final equations, Figure 3 presents the plots of the observed versus predicted data by the final equations for each microorganism. The difference between a point on the graph and the line of equivalence is a measure of the accuracy of the corresponding estimation. The R^2, RMSE, accuracy (A_f), and bias (B_f) factors from each prediction were also indicated in the figures. The calculated values demonstrated that, in general, the final equations accurately predicted the UV-H inactivation of all microorganisms without observing over or under predictions. Therefore, the developed final equations would be adequate to compare the UV-H resistance of the different microorganisms and to define the processing conditions (process criteria) to achieve a certain level of pathogenic microbial inactivation (performance criteria), as will be discussed later on.

It has been demonstrated that the improvement of UV-H inactivation was due to the occurrence of a synergistic lethal

Table 2: Parameters of the secondary models resulting after fitting Albert and Mafart's (2) and Mafart's models (3) to the data representing the relationship between the Sl and K_{max} from UV-H survival curves of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* from Table 1 against the treatment, respectively. Values in parentheses represent the standard errors of the means. Letters a, b, and c indicate statistically significant differences ($P \leq 0.05$).

(a) Shoulder length secondary model

Microorganism	δ	p	Sl_0	Sl_{res}	R^2	RMSE
Escherichia coli	56.81 (0.28)^a	21.46 (2.99)^a	0.70 (0.02)^a	0.00 (0.02)^a	0.996	0.027
Salmonella Typhimurium	56.69 (0.42)^a	16.52 (2.76)^b	0.70 (0.03)^a	0.00 (0.02)^a	0.992	0.034
Listeria monocytogenes	57.05 (0.16)^a	24.06 (2.71)^a	1.05 (0.03)^b	0.00 (0.02)^a	0.998	0.032
Staphylococcus aureus	57.45 (0.70)^a	12.18 (1.72)^b	0.57 (0.02)^c	0.00 (0.03)^a	0.993	0.025

(b) K_{max} secondary model

Microorganism	δ	p	K_{max0}	R^2	RMSE
Escherichia coli	69.22 (0.39)^a	8.39 (0.57)^a	3.70 (0.07)^a	0.998	0.071
Salmonella Typhimurium	63.11 (1.27)^b	10.95 (3.44)^a	2.76 (0.39)^{bc}	0.981	0.501
Listeria monocytogenes	67.08 (0.79)^a	9.44 (1.68)^a	2.52 (0.14)^b	0.989	0.151
Staphylococcus aureus	62.25 (1.68)^b	8.02 (2.25)^a	3.49 (0.53)^{ac}	0.985	0.546

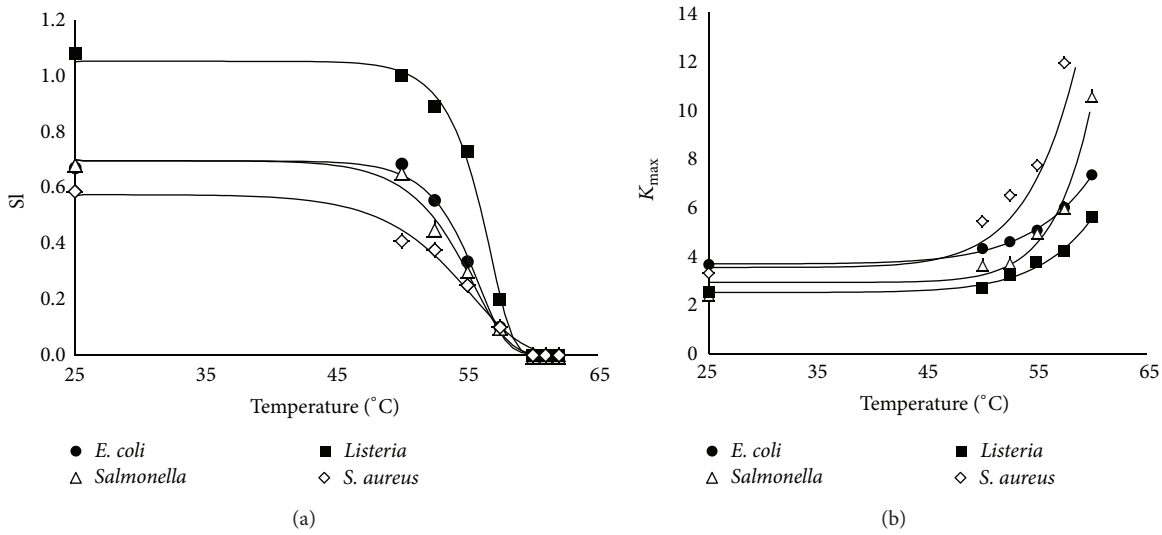

Figure 2: Relationships between temperature and Sl (a) or K_{max} (b) values obtained after the fitting of Geeraerd's model (1) to the UV-H inactivation data of *E. coli* (●), *Salmonella* Typhimurium (△), *L. monocytogenes* (■), and *S. aureus* (◇) in chicken broth. Solid lines represent the fitting curves for Sl and K_{max} calculated from Albert and Mafart's (2) and Mafart's equation (3), respectively.

effect and that the magnitude of this effect increased when the treatment temperature was raised to a threshold value [8, 10]. Above this temperature, thermal lethal effects began to predominate over UV lethality, and UV-H synergism was reduced to the point of disappearing, whereby microbial death was then exclusively due to heat. Prior studies on the combined UV-H treatment of apple juice have shown that *E. coli* STCC 4201 displayed the maximum UV-H synergism at a treatment temperature of about 55°C; above this temperature, the synergism decreased until it disappeared at 60.0°C when UV-H and heat survival curves overlapped [10]. Therefore, to take advantage of the combined UV-H process, the treatment temperature should be limited to temperatures below the intersection of UV-H and heat lethality. This requires knowledge of the heat resistance abilities of target microorganisms and their thermodependence. Therefore, the next step was to investigate the heat resistance of pathogenic microorganisms in chicken broth.

3.2. Microbial Inactivation by Heat Treatments in Chicken Broth. To evaluate the contribution of heat to the lethal effect of the combined UV-H treatment, experiments on the heat resistance of all the investigated microorganisms were carried out also in chicken broth. The resulting survival curves did not follow first order kinetics and presented an initial lag phase (shoulder). *E. coli* was the only exception, showing an inactivation curve with a log-linear behavior. The inactivation data were fitted by Geeraerd's model (primary model; (1)). The resulting values for the heat resistance parameters (Sl and K_{max}) were included in Table 3. As observed, Sl values decreased with temperature, becoming zero at the highest

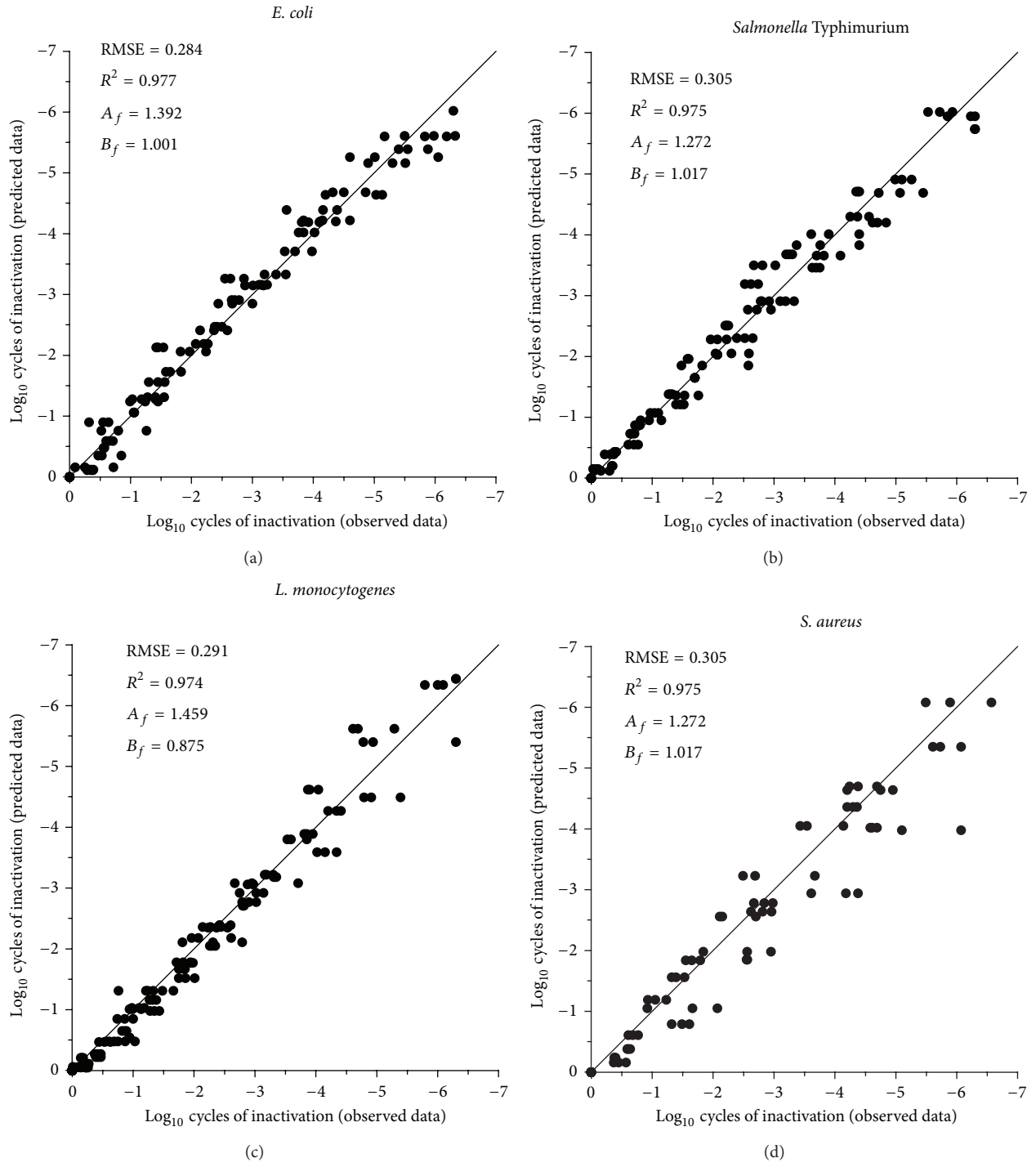

Figure 3: Correlation between observed and predicted data obtained from the tertiary models for *E. coli* (a), *Salmonella* Typhimurium (b), *L. monocytogenes* (c), and *S. aureus* (d) when treated by UV-H process. R_2, RMSE, accuracy (A_f), and bias (B_f) factors from each prediction are also indicated in the figures.

temperatures. That is, the shoulders disappeared proportionate to temperature, as was observed in the UV-H treatments. The relationship between Sl and temperature also followed the Weibullian distribution and was described by Albert and Mafart's equation (2). In the case of K_{max} values, they increased with temperature following an exponential relationship. These relationships (secondary models) allowed for the obtention of the corresponding kinetic parameters showed in Table 4. As observed, the shoulder length (Sl value) was higher for *S. aureus*. However, it rapidly decreased with temperature differently to *L. monocytogenes*, which was less influenced by the temperature (P value). Concerning the

Table 3: Resistance parameters obtained from the fit of heat inactivation data of *E. coli*, *Salmonella* Typhimurium, *S. aureus*, and *L. monocytogenes* at different temperatures in chicken broth by Geeraerd's model (1). Estimated standard deviations (SD) of the means are shown in parentheses. Letters a, b, c, and d indicate statistically significant differences ($P \leq 0.05$) among Sl and K_{max} values of heat survival curves for each microorganism at different temperatures.

Microorganism	Temperature (°C)	Sl (min)	K_{max} (min^{-1})	R^2	RMSE
Escherichia coli	55.6	—	0.52 (0.02)[a]	0.981	0.204
	58.1	—	1.33 (0.04)[b]	0.993	0.203
	60.6	—	3.65 (0.11)[c]	0.991	0.122
	62.6	—	17.49 (1.02)[d]	0.992	0.156
Salmonella Typhimurium	55.6	0.41 (0.71)[a]	0.49 (0.11)[a]	0.994	0.024
	58.1	0.19 (0.12)[b]	3.00 (0.16)[b]	0.993	0.196
	60.6	0.00 (0.00)[c]	7.03 (0.66)[c]	0.995	0.188
	62.6	0.00 (0.00)[c]	27.97 (1.23)[d]	0.994	0.157
Listeria monocytogenes	55.6	0.80 (0.15)[a]	1.27 (0.11)[a]	0.995	0.105
	58.1	0.81 (0.27)[a]	2.22 (0.23)[b]	0.983	0.153
	60.6	0.80 (0.19)[a]	3.47 (0.11)[c]	0.992	0.204
	62.6	0.47 (0.00)[b]	5.81 (0.15)[d]	0.995	0.165
Staphylococcus aureus	53.1	1.30 (0.04)[a]	2.24 (0.03)[a]	0.996	0.082
	55.6	0.12 (0.09)[b]	3.48 (0.04)[b]	0.983	0.204
	58.1	0.00 (0.00)[c]	5.93 (0.31)[c]	0.995	0.163

Table 4: Secondary model parameters calculated after plotting Sl and K_{max} values obtained from heat survival curves of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* against treatment temperature and fitting by Albert and Mafart's equation (2) and log-linear regressions, respectively. Values in parentheses represent the standard errors of the means.

(a) Shoulder length secondary model

Microorganism	δ (min)	p	Sl_0 (min)	Sl_{res} (min)	R^2	RMSE
Escherichia coli	—	—	—	—	—	—
Salmonella Typhimurium	59.00 (0.07)[a]	63.00 (7.23)[a]	0.43 (0.00)[a]	0.00 (0.02)[a]	0.999	0.048
Listeria monocytogenes	63.80 (0.11)[b]	39.04 (3.46)[b]	0.87 (0.01)[b]	0.00 (0.02)[a]	0.998	0.099
Staphylococcus aureus	55.26 (0.03)[c]	96.67 (8.66)[c]	1.32 (0.00)[c]	0.00 (0.03)[a]	0.999	0.063

(b) K_{max} secondary model

Microorganism	Slope (min^{-1})	Intercept (min)	R^2	RMSE
Escherichia coli	0.212 (0.027)[a]	−12.135 (1.602)[a]	0.969	0.425
Salmonella Typhimurium	0.240 (0.024)[a]	−13.602 (1.405)[a]	0.981	0.088
Listeria monocytogenes	0.093 (0.004)[b]	−5.042 (0.244)[b]	0.996	0.018
Staphylococcus aureus	0.084 (0.021)[b]	−4.138 (0.121)[b]	0.999	0.009

relationships of the inactivation rate, K_{max} values of *E. coli* and *Salmonella* Typhimurium showed a similar thermodependence (no significant differences among the slope values), although it was somewhat higher than those of both Gram positive microorganisms, whose thermodependence varied in a parallel manner. This means that the velocity of death of *L. monocytogenes and S. aureus* was affected to a lesser extent by temperature changes than that of *E. coli* and *Salmonella* Typhimurium.

To predict the heat inactivation in chicken broth for each studied microorganism, final equations were developed by including the obtained secondary models for Sl and K_{max} in Geeraerd's equation (primary model). When comparing the observed inactivation data at different times and temperatures from those predicted by the final equations, R^2 values ranged from 0.932 to 0.987, RMSE from 0.158 to 0.386, A_f from 1.132 to 1.444, and B_f from 0.830 to 1.207, indicating an adequate goodness of the fits. Similar to the UV-H final equations, the developed final equations for heat would be adequate to compare the heat resistance of the different microorganisms; define the process criteria to achieve a certain performance criteria; and compare the microbial lethal effectiveness of both the heat and UV-H treatment processes.

3.3. Process Criteria for 5-Log_{10} Reductions of Foodborne Microbial Pathogens in Chicken Broth. Figure 4 shows the logarithm of the treatment time and UV dose necessary to inactivate 5-Log_{10} cycles of *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus* at temperatures between

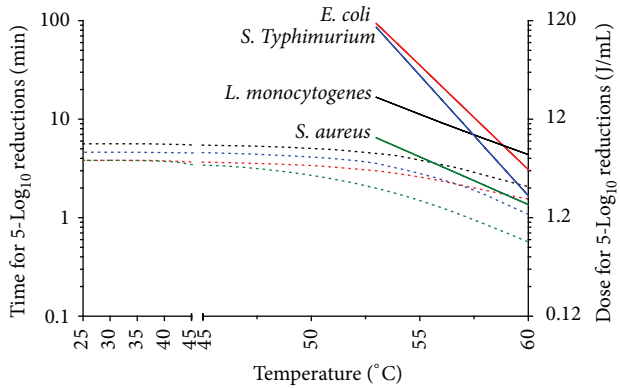

FIGURE 4: Times and UV doses required to achieve 5-Log_{10} reductions by UV-H (dotted lines) and heat treatments (continuous lines) at different temperatures for *E. coli* (red line), *Salmonella* Typhimurium (blue line), *L. monocytogenes* (black line), and *S. aureus* (green line) in chicken broth.

25°C and 60.0°C in chicken broth. UV doses displayed on the secondary *OY* axis were calculated from the trend line equation that resulted from plotting the treatment time against the corresponding UV dose for each reactor. In our facility, this relationship was UV dose = 1.0837 ∗ (time) + 0.0213 (R^2 = 0.999). For comparison reasons, thermal death time (TDT) curves for each microorganism have also been included showing the relationship between the time for 5-Log_{10} reductions and the heating temperature. In the case of the heat treatments, all TDT curves showed a log-linear profile from which z values were deduced (temperature increase for reducing the treatment time 10-fold) at 4.7°C, 4.2°C, 10.8°C, and 11.8°C for *E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus*, respectively. These values are in the range of what other authors have obtained for fruit juices [30, 36–38], although slightly higher in the case of the Gram positive bacteria. Based on these data, *E. coli* was the most heat resistant bacterium when the temperature was increased to about 58.8°C, the temperature at which the TDT curves of *E. coli* and *L. monocytogenes* intersected. Above this temperature, *L. monocytogenes* became the most thermotolerant microorganism.

Concerning the UV-H treatments, at temperatures over 50°C the treatment time for 5-Log_{10} reductions of all microorganisms decreased with temperature and followed a concave downward profile. The 5-Log_{10} reductions time varied with the treatment temperature in a way that paralleled that of the thermodependence of K_{max} of the UV-H survival curves (Figure 2 and Table 1). *L. monocytogenes* was the most UV-H resistant microorganism at all studied temperatures. UV doses of 6.1 J/mL at room temperature would permit the 5-Log_{10} reduction of any of the investigated pathogenic microorganisms in 5.6 minutes, a level of inactivation that is difficult to achieve with other technologies at the investigated temperatures, treatment times, and energy costs in products like broths. The UV dose and time required to achieve 5-Log_{10} reductions in the target pathogen (*L. monocytogenes*) by UV treatment at room temperature would be reduced by 19.7% at 53°C. A more radical improvement was attained by raising the temperature to 55.0, 57.5, and 60.0°C at which the UV dose and time needed to achieve the performance criterion for *L. monocytogenes* was reduced by 30.1, 44.8, and 62.9%, respectively.

When comparing UV-H and heat treatments, the application of UV-C light at moderate temperatures noticeably reduced the heat treatment time for 5-Log_{10} reductions of tested microorganisms in chicken broth. This reduction in time was greater the lower the temperature of the UV-H treatment was applied. When UV-H treatments were applied at 53°C, 4.5 and 93.5 minutes of the UV-C and heat treatments would be required, respectively, that is, a 20-fold time reduction of the heat processing time. This time reduction of the heat treatments was of 9-, 4-, and 2-fold when the temperature was 55.0, 57.5, and 60.0°C, respectively. Over 60°C, the microbial inactivation due to heat would be greater, thereby reducing the synergistic lethal contribution of UV light and achieving a temperature over which inactivation would be only due to heat.

3.4. Conclusions. This study has investigated the lethal microbial effects of UV-C, heat, and UV-H on the different pathogenic microorganisms of reference (*E. coli*, *Salmonella* Typhimurium, *L. monocytogenes*, and *S. aureus*) when treated in chicken broth. Mathematical equations were developed that enabled a comparison between the investigated microorganisms' levels of resistance to the different applied technologies. This defined the target microorganism and established the process criteria (UV dose, time, and temperature) required for 5-Log_{10} reductions of the four pathogens in chicken broth. With all technologies, 5-Log_{10} reductions were obtained for all investigated microorganisms. However, depending on the technology and the target microorganisms, the required time to achieve that performance criterion noticeably varied. For UV treatments (at room or at moderate temperatures), *L. monocytogenes* was the most resistant microorganism, but for heat treatments up to temperatures of 59°C, *E. coli* was. Over this temperature, *L. monocytogenes* was again the most resistant bacteria in chicken broth. On the other hand, UV-C light applied even at room temperature resulted in a technology to control foodborne pathogens in chicken broth that was very promising. However, the time of treatment at this temperature could possibly be too long from a practical point of view. The application of UV-H permitted a reduction in this processing time, which happened to be 20- to 2-fold lower than the corresponding to heat treatments at the same temperature and with an extra energetic cost varying from 6.10 J/mL to 2.26 J/mL. These results indicated that combining UV-C light with mild temperatures permitted a certain performance criterion to be achieved by means of lower UV doses and treatment times than those needed for UV or heat treatments applied to chicken broth alone. The fact that this food product is not considered suitable for being treated by UV light due to its high turbidity and absorption coefficient enhances the importance of the aforementioned results.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Salmonella Typhimurium and *Salmonella* Sofia: Growth in and Persistence on Eggs under Production and Retail Conditions

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Salmonellosis in Australia has been linked to eggs and egg products with specific serotypes associated with outbreaks. We compared attachment to and survival on egg shells and growth in eggs of two *Salmonella* serotypes, an egg outbreak associated *Salmonella* Typhimurium and a non-egg-associated *Salmonella enterica* ssp. II 1,4,12,27:b:[e,n,x] (*S.* Sofia). Experiments were conducted at combinations of 4, 15, 22, 37 and 42°C. No significant differences occurred between the serotypes in maximum growth rates, which were significantly greater ($P < 0.001$) in egg yolk (0.427 $\log_{10}$ CFU/mL/h) compared to whole egg (0.312 $\log_{10}$ CFU/mL/h) and egg white (0.029 $\log_{10}$ CFU/mL/h). Attachment to egg shells varied by time (1 or 20 min) and temperature (4, 22 and 42°C), with *S.* Typhimurium isolates attaching at higher levels ($P < 0.05$) than *S.* Sofia after 1 min at 4°C and *S.* Typhimurium ATCC 14028 attaching at higher ($P < 0.05$) levels at 22°C. Survival on egg shells was not significantly different across isolates. *Salmonella* serotypes behaved similarly regarding growth in egg contents, attachment to egg shells and survival on eggs, indicating that other factors more likely contributed to reasons for *S.* Typhimurium being implicated in multiple egg-associated outbreaks.

1. Introduction

For organisms to contaminate products or surfaces along the food chain, they must first attach. This initial attachment may take the form of loosely or firmly attached cells. Loosely attached cells are readily removed but firmly attached cells are more difficult to remove and may eventually, under the right conditions, grow to form biofilms, usually comprising a microflora of mixed populations closely associated with organic matter, assisting in the persistence of pathogenic bacteria. There are a number of potential contamination points during egg production and processing. For example, in countries where *Salmonella enterica* subsp. *enterica* serotype Enteritidis (SE) is found, infection of the laying hen may lead to internal contamination of eggs prior to oviposition (vertical transmission) or alternatively eggs may become contaminated with *Salmonella* immediately following lay, due to contact with feces or fecally contaminated laying material (horizontal transmission) [1]. The process of washing and grading eggs may also contaminate eggs via cross contamination from other eggs, contact with contaminated surfaces, or through contaminated wash water. Further contamination may occur at the consumer end of the food chain due to cross contamination from other products during storage, handling, and food preparation.

In Canada, the USA, and the EU, foodborne outbreaks due to *Salmonella* contamination of eggs are largely due to SE [2]. In Australia, eggs are predominantly contaminated with *Salmonella* Typhimurium (STM) [2]. With the predominance of SE in eggs in other parts of the world, much of the focus of international research into how *Salmonella* can be transmitted by eggs has focused on SE. The survival of

Salmonella on the surface of eggs and subsequent penetration into the egg show a range of variable results as described in previous reviews [1, 3, 4] again with a greater focus on SE.

The predominant *Salmonella* serotypes detected in *Salmonella* infections in Australia can vary between the states and territories [5]. Numerous outbreaks of salmonellosis associated with eggs and egg products have been caused by specific, recurring serovars and phage types of *Salmonella*, including *S.* Typhimurium phage type 135 (PT135) between 2006 and 2010 [5, 6]. In particular, this phage type has been attributed to multiple salmonellosis outbreaks associated with egg consumption in Tasmania from 2005 to 2008 [7] and more recently was identified as the cause of an egg-associated salmonellosis outbreak in Canberra in 2012 [8]. A draft genome sequence of isolates associated with the Tasmanian outbreak as well as the short-term microevolution of these isolates has been published [9, 10].

This study utilized a known egg-related outbreak strain of *S.* Typhimurium PT135, a laboratory reference strain of *S.* Typhimurium, and a nonegg-associated, meat chicken-specific serovar of *S.* Sofia to test the hypothesis that an egg outbreak-associated *Salmonella* strain is better at attaching to, growing in, and/or surviving on eggs than nonegg-associated *Salmonella*. Experiments were conducted to determine attachment, survival on egg shell, and growth in egg contents in experiments at various temperatures relevant to egg production. These included 4°C, a common refrigeration temperature, 15°C, which is a production storage temperature recommended by the Australian Egg Corporation Limited [11], 22°C, ambient temperature, 37°C, which is an optimum growth temperature for *Salmonella,* and 42°C, which is the body temperature of chickens and is within the recommended egg wash temperature range [11].

2. Materials and Methods

2.1. Egg Source. Extra large eggs produced by caged hens were sourced from either local supermarkets, a single farm through farm gate sale or by arrangement with a central processor, and were stored at 4°C until use. Supermarket and farm gate sale eggs were washed and used in the growth and attachment studies. Eggs used for survival on egg shell studies were unwashed and sourced from a single farm by arrangement with a central processor.

2.2. Bacterial Strains. The three *Salmonella* strains assessed in this study were *S.* Typhimurium PT135 (STM), an egg outbreak strain isolated from a clinical sample, the reference strain *S.* Typhimurium ATCC 14028 (RS), and *S.* Sofia 1296a (SS) isolated from a meat chicken carcass. All three strains were used to assess the growth in and attachment to eggs. The STM and SS strains were used to assess the survival of *Salmonella* on eggs under potential consumer storage and retail conditions.

2.3. Growth in Eggs. The growth or survival of the *Salmonella* isolates was assessed individually in egg yolk, egg white, and whole egg. Each egg component was tested at 15, 22, and 37°C. Egg yolk and whole egg trials were conducted until the cultures reached stationary phase or up to 44 days. Counts of *Salmonella* in egg whites were followed for up to 35 days. All experiments were performed at least in duplicate.

The outside of the eggs was sanitized prior to cracking by immersion in 80% ethanol for two minutes followed by drying via evaporation in a laminar flow cabinet. Eggs were aseptically cracked and separated and egg components tipped into sterile 50 mL Falcon tubes (PLP, Blackburn, Victoria, Australia). Each egg yolk trial used two egg yolks. The egg white and whole egg trials utilized the components from one egg each. Egg whites were gently mixed using a sterile whisk to break up the egg white prior to the trials. Whole eggs were also gently mixed with a sterile whisk prior to use.

Salmonella strains were grown in Tryptone Soya Broth (Oxoid, Basingstoke, England) at 37°C for 20 h and then diluted in Maximum Recovery Diluent (Oxoid) so that the addition of 0.1 mL of inoculum to the egg resulted in approximately 5×10^2 CFU/mL of egg content, determined by pre-trial inoculum studies, for the egg yolk and whole egg preparations at all three temperatures. The 10^2 CFU/mL inoculum was selected as it is close to the limit of detection on 0.1 mL spread plates. The egg white treatments had a starting inoculum of approximately 5×10^4 CFU/mL at all three temperatures with the egg white treatment at 15°C also tested at the lower inoculum level of 5×10^2 CFU/mL. A higher inoculum level was used for the egg whites so that both growth and death could be monitored. The inoculum was mixed with the egg contents by vortexing. Uninoculated egg contents were used as the controls.

All samples were tested after inoculation (T0) and then at various intervals depending on the temperature and egg component. At each sampling period, the tubes were gently mixed by inversion 25 times. Serial dilutions of 0.2 mL were made in 1.8 mL of Buffered Peptone Water (BPW; Oxoid) and spread plated (0.1 mL) in duplicate onto Tryptone Soya Agar (TSA; Oxoid). An enrichment of 0.2 mL sample in 1.8 mL BPW was also performed when the counts fell below the limit of detection on the plates (10 CFU/mL). The limit of detection in the broth was 5 CFU/mL. All plates and enrichments were incubated at 37°C for 24 ± 2 h and then counted. Enrichments were streaked onto TSA and incubated at 37°C for 24 ± 2 h. After counting, three colonies were selected from the TSA plates of each isolate at the beginning and end of each trial and confirmed as *Salmonella* using a *Salmonella* latex test kit (Oxoid).

2.4. Attachment to Egg Shells. The attachment of *Salmonella* to egg shells immediately following exposure for either one or 20 min was assessed on eggs sourced from a retail outlet as well as from a single farm through farm gate sale at 4, 22, and 42°C. Eggs were equilibrated at the test temperature for a minimum of 2 h before inoculation. The inoculum was prepared by growing the strains individually in 1 L of BPW at 37°C for 18 ± 2 h without shaking. The BPW was centrifuged at 4500 ×g for 25 min at 4°C and cells were resuspended in 1 L of phosphate buffered saline (PBS; Sigma, Australia) that had been previously held overnight at 4, 22, or 42°C, as required. Eggs were dipped in the inoculum (8.66 ± 0.16 CFU/mL)

for either one or 20 min, removed, and rinsed twice by gentle swirling for 5 s in 150 mL PBS equilibrated to the same temperature as used in the attachment assay. Individual eggs were then placed in a stomacher bag (Sarstedt, Australia) and massaged in the bag in 50 mL of BPW for 3 min to detach the bacteria. Each experiment was conducted in duplicate and three eggs were used for each attachment assay.

Enumeration of the *Salmonella* was conducted by spread plating 0.1 mL of suitable serial dilutions onto TSA. Plates were incubated at 37°C for 18 ± 2 h before the number of colonies was counted and recorded. A total of 10 colonies were confirmed as *Salmonella* from TSA plates using a *Salmonella* latex test kit (Oxoid). A total of three eggs from each tray were used as control eggs. These eggs were placed individually in stomacher bags and gently but vigorously rubbed and shaken in 50 mL of BPW. A portion of BPW was spread plated onto TSA and incubated at 30°C for 72 h to obtain a total viable count (TVC). Control eggs were also examined for the presence of *Salmonella* by plating onto xylose lysine deoxycholate (XLD, Oxoid) agar and enriching the remaining BPW at 37°C for 18±2 h. A 0.1 mL portion was inoculated into Rappaport-Vassiliadis Soya Peptone Broth (RVS, Biomerieux, France) and incubated for 24 h at 42°C. The RVS was streaked onto XLD and incubated at 37°C for 24 h. XLD plates were examined for typical *Salmonella* colonies.

2.5. Survival on Egg Shells. The STM and SS strains were assessed for survival on egg shells under retail conditions at 4 and 22°C stored in clean, unused, half-dozen cardboard egg containers wrapped in brown paper. The relative humidity (RH) inside the cartons of six eggs ranged from 88 to 100% at 4°C and from 38 to 55% at 22°C.

Cultures of STM and SS were grown in Heart Infusion Broth (Oxoid) for 24 h at 37°C and viable counts determined on pour plates (1 mL) of Yeast Extract Agar [12] followed by incubation for 48 h at 37°C. Overnight broth cultures were diluted 1 : 100 in BPW prewarmed at 42°C and held at this temperature for 30 min, prior to pipetting two × 50 μL (approximately 6 $\log_{10}$ CFU) aliquots to two × 1 cm^2 areas (delineated by marker pen ink) on the surface of a prewarmed (42°C) unwashed egg, placed on a sterile wire mesh rack inside a Class II Biological Safety Cabinet and allowed to dry for up to 3 h. After the surface inoculums had dried, the uninoculated area of the eggs outside the delineated areas was swabbed with 70% ethanol and allowed to evaporate until dry so as to reduce the level of naturally occurring microflora outside of this area.

Over the duration of the four-week storage trial, five eggs were tested at week 0 (T0), then after 1, 2, and 4 weeks. Survival of *Salmonella* on individual eggs was determined by placing each egg in a sterile stomacher bag containing 10 mL BPW and held at 4°C for 16 h to soften cuticle material and then subjected to abrasion with a sterile swab to assist in removing bacterial cells from the egg surface. The BPW was then dispensed as a five tube Most Probable Number (MPN) over a 5 $\log_{10}$ dilution range using a replicate of five eggs. The remainder of the BPW not used in determination of MPN was made up to 10 mL and used to enrich the intact egg to detect surviving *Salmonella* that may have remained attached to the egg. The BPW MPN tubes and whole egg BPW enrichments were incubated for 24 h at 37°C. Following incubation, 0.1 mL was placed into 10 mL RVS (BBL Phytone Peptone, BD, Maryland, USA) and incubated for 24 h at 42°C. RVS broths were then streaked onto XLD agar and incubated for 24 h at 37°C. Colonies with typical *Salmonella* morphology on XLD were subjected to biochemical, serological agglutination and phage-typing analyses to confirm respective isolates as SS or STM. MPN values were calculated for both visible turbidity (indicative of a TVC of aerobic microorganisms) and *Salmonella*, scored using a three dilution range of five tubes. Uninoculated eggs were swabbed with 70% ethanol and treated as above for use as controls.

2.6. Statistical Analysis. All counts were transformed to $\log_{10}$ CFU/mL prior to statistical analysis. A line of best fit was constructed from Microsoft Excel scatter plots using a polynomial equation for the growth in egg total counts of each isolate. The DMFit predictive models (http://www.combase.cc/index.php/en/resources) were used to analyse the $\log_{10}$ CFU/mL counts for the growth in egg data sets and generate the maximum rate (CFU/mL/h). Analysis of variance (ANOVA) was conducted on the maximum rate and survival on egg shell storage trial data (time, temperature, and strain) using GenStat 13.1 (VSN International Ltd., Hemel, UK). The mean $\log_{10}$ CFU/mL count of each of six eggs (three eggs in duplicate) was used to compare attachment levels between strains, temperature, time, and source of egg (retail or farm gate) using Minitab 16 (Minitab Inc., Minnesota, USA). A one-way analysis of means using Tukey's method for multiple comparisons was performed on pairs of data sets using Minitab. Results were considered significant at $P < 0.05$.

3. Results

3.1. Growth in Eggs. The growth rates of STM, SS, and RS were not significantly different ($P > 0.05$) in any of the egg components at any temperature, showing that the outbreak strain of *Salmonella* Typhimurium did not grow faster than the other *Salmonella* isolates and suggesting that multiple *Salmonella* species could grow quickly in egg contents if conditions were suitable. As the growth rates between the isolates were not significantly different ($P > 0.05$), the results were pooled for each egg component and temperature. The mean maximum growth rates of *Salmonella* were determined in each of the egg components at different temperatures (Table 1) with the greatest growth rates occurring at higher temperatures for each egg component. Growth was significantly greater ($P < 0.001$) in the egg yolk, than in the whole egg or egg white at all temperatures. The highest maximum growth rates occurred in egg yolks at 37°C and ranged from 0.7735 to 0.9064 $\log_{10}$ CFU/mL/h. The lowest maximum growth rates occurred in the egg whites with negative growth rates (ranging from −0.0138 to 0.0187 $\log_{10}$ CFU/mL/h) observed in egg whites incubated at 15°C. Inoculation level and isolate did not play a role in determining whether *Salmonella* counts increased or decreased in the egg white at 15°C. At 22°C in the egg white, the majority of growth rates were positive and

Table 1: Mean maximum growth rates (CFU/mL/h) of *Salmonella* isolates with the higher inoculum level in the egg white.

Temperature	SS[1]			STM[2]			RS[3]			All isolates combined[4]		
	Egg yolk	Whole egg	Egg white	Egg yolk	Whole egg	Egg white	Egg yolk	Whole egg	Egg white	Egg yolk	Whole egg	Egg white
15°C	0.116	0.093	0.005	0.115	0.099	−0.007	0.112	0.094	−0.001	0.114^{aA}	0.095^{aA}	-0.001^{bA}
22°C	0.324	0.211	0.022	0.323	0.238	0.023	0.328	0.237	0.040	0.325^{aB}	0.228^{bB}	0.028^{cAB}
37°C	0.821	0.613	0.095	0.865	0.614	0.076	0.840	0.610	0.010	0.842^{aC}	0.612^{bC}	0.060^{cB}
All temperatures	0.420	0.306	0.041	0.427	0.314	0.016	0.434	0.317	0.031	0.427^{a}	0.312^{b}	0.029^{c}

[1]SS: *S.* Sofia 1296a; [2]STM: *S.* Typhimurium PT135; [3]RS: *S.* Typhimurium ATCC 14028.
[4]There were no significant differences between the isolates individually ($P > 0.05$); therefore the isolates were combined to assess the effects of temperature and egg component. Different lower case letters within rows are significantly different ($P < 0.001$). Different upper case letters within columns are significantly different ($P < 0.001$).

ranged from −0.005 to 0.1015 $\log_{10}$ CFU/mL/h. Then at 37°C in the egg white, the majority of growth rates were again positive but ranged from −0.1227 to 0.1434 $\log_{10}$ CFU/mL/h.

All of the *Salmonella* isolates grew to stationary phase (10^8-10^9 CFU/mL) in the egg yolk and whole egg. Stationary phase was only achieved in six of the 24 higher starting inoculum egg white experiments. The time to reach stationary phase in the egg yolk was three days at 15°C, 26 h at 22°C, and 9 to 10 h at 37°C for each of the strains. In the whole egg stationary phase was reached in four days at 15°C, 34 h at 22°C, and 12 to 16 h at 37°C. The isolates did not grow in the egg white compared to the yolk and whole egg as well and there was more variation between replications in the egg white. The survival of *Salmonella* in egg white was tested at two inoculum levels at 15°C. The largest maximum standard deviation between replicate time points was seen in the egg white at 15°C at the higher inoculum for STM. In the high inoculum egg white at 15°C, only one isolate (STM) reached 10^8 CFU/mL after 34 days in one replicate experiment, whereas the numbers of all of the other replicates and isolates either remained steady or declined over time. All of the isolates decreased in number in egg white when the lower inoculum was used and dropped to near or below the limit of detection after 35 days. At 22°C, the maximum standard deviations between replicate time points were 1.5- to 3-fold greater in the egg white than in the yolk and whole egg for all three strains. Reproducibility between trials in egg white at 22°C was variable with the time to grow to stationary phase different between trials and between strains, ranging from three to 30 days or longer. At 37°C, the level of *Salmonella* in egg white remained relatively unchanged for 16 h, but after six days the counts of STM had dropped by one $\log_{10}$ and the viable count for RS had dropped below the limit of detection (5 CFU/mL), while SS counts remained unchanged. *Salmonella* was not detected in the control eggs.

3.2. Attachment to Eggs. No *Salmonella* was detected on control eggs. All TVC were <410 CFU/mL. All *Salmonella* isolates attached to eggs at similar levels with the highest attachment (5.85 $\log_{10}$ CFU/mL) by RS at 22°C for 20 min on farm gate sourced eggs, while the lowest level of attachment (3.77 $\log_{10}$ CFU/mL) was by SS at 4°C for 1 min on farm gate sourced eggs (Tables 2 and 3). STM and SS attached at significantly ($P < 0.05$) lower levels than RS at 22°C on farm eggs. STM and RS attached at significantly ($P < 0.05$) higher levels than SS after 1 min at 4°C on farm eggs.

Within all combinations of strain, egg source, and temperature, the attachment levels after 1 min were lower than after 20 min. However, this difference was significant ($P < 0.05$) only for strains SS and RS when attached to farm eggs at 22°C. Strain differences were noted only on farm sourced eggs for a number of combinations of time/temperature. At 22°C STM attached significantly lower than RS on farm eggs, but not at a significantly ($P > 0.05$) different level to SS after 20 min. STM and RS attached at significantly ($P < 0.05$) higher levels after 1 min than SS on farm eggs at 4°C. There was a significantly ($P < 0.05$) lower level of attachment of SS after 20 min compared to RS at all temperatures. The source of eggs either retail or farm did not affect the level of attachment of either STM or RS for any time/temperature combination. However the source of eggs, retail (5.26 $\log_{10}$ CFU/mL) or farm gate (4.68 $\log_{10}$ CFU/mL), had a significant ($P < 0.05$) effect on the level of attachment of SS at 42°C after 1 min.

Temperature also had an interactive effect on the level of attachment of some strain, time, and egg source combinations. On retail eggs at 4°C, SS attached at a significantly ($P < 0.05$) lower level than at 42°C after 1 min of attachment time. On farm gate sourced eggs, SS at 4°C again attached at significantly ($P < 0.05$) lower levels than at 42°C, after 1 min but not after 20 min. *Salmonella* was not detected on any control eggs.

3.3. Survival on Eggs. The TVC of both eggs inoculated with *Salmonella* and the uninoculated control eggs stored at 4°C showed a decreasing trend in the first two weeks of storage with an approximately 2.0 $\log_{10}$ MPN/cm decrease after two weeks. In contrast, eggs stored at 22°C challenged with SS maintained viable count levels throughout four-week storage. At the same temperature, eggs challenged with STM showed a similar trend, although a decrease of about 1.0 $\log_{10}$ MPN/cm was observed at week four.

The results for recovery of *Salmonella* from egg surfaces indicate that neither strain of SS or STM persisted past four weeks. SS was recovered from eggs by either MPN or selective enrichment procedures applied to intact eggshells and was isolated at both storage conditions at week two (four out of five eggs) whereas STM could only be recovered after one week of storage (two out of five eggs) at 22°C. At 4°C, STM

Table 2: The level of attachment of each isolate for retail eggs (± standard deviation) at 4, 22, and 42°C after 1 or 20 min of attachment.

Temperature	SS[1]		STM[2]		RS[3]	
	1 min	20 min	1 min	20 min	1 min	20 min
4°C	4.43 ± 0.36^{aA}	4.78 ± 0.29^{abA}	5.01 ± 0.08^{abA}	5.14 ± 0.34^{bA}	4.96 ± 0.31^{abA}	5.36 ± 0.24^{bA}
22°C	4.78 ± 0.80^{aAB}	5.24 ± 0.18^{aA}	4.88 ± 0.35^{aA}	5.19 ± 0.23^{aA}	4.99 ± 0.23^{aA}	5.25 ± 0.46^{aA}
42°C	5.26 ± 0.28^{aB}	5.32 ± 0.15^{aA}	4.82 ± 0.26^{aA}	5.1 ± 0.15^{aA}	4.85 ± 0.28^{aA}	5.28 ± 0.42^{aA}

Different lower case letters within rows are significantly different ($P < 0.05$). Different upper case letters within columns are significantly different ($P < 0.05$).
[1]SS: *S.* Sofia 1296a; [2]STM: *S.* Typhimurium PT135; [3]RS: *S.* Typhimurium ATCC 14028.

Table 3: The level of attachment of each isolate for farm eggs (± standard deviation) at 4, 22, and 42°C after 1 or 20 min of attachment.

Temperature	SS[1]		STM[2]		RS[3]	
	1 min	20 min	1 min	20 min	1 min	20 min
4°C	4.14 ± 0.25^{aA}	4.64 ± 0.19^{abA}	4.72 ± 0.39^{bcA}	5.11 ± 0.15^{bcdA}	5.10 ± 0.16^{bcdA}	5.23 ± 0.16^{cdeA}
22°C	4.16 ± 0.18^{aAB}	4.85 ± 0.12^{bcA}	4.60 ± 0.19^{abA}	5.00 ± 0.20^{bcA}	4.83 ± 0.14^{bcA}	5.75 ± 0.10^{dA}
42°C	4.68 ± 0.19^{aB}	4.81 ± 0.24^{abA}	5.06 ± 0.65^{abcA}	5.09 ± 0.26^{abcA}	5.04 ± 0.12^{abcA}	5.56 ± 0.21^{cA}

Different lower case letters within rows are significantly different ($P < 0.05$). Different upper case letters within columns are significantly different ($P < 0.05$).
[1]SS: *S.* Sofia 1296a; [2]STM: *S.* Typhimurium PT135; [3]RS: *S.* Typhimurium ATCC 14028.

was not detected on any of the five eggs after one week. Evaluating the viable level of *Salmonella* recovered using an MPN procedure also showed that there was rapid decline in the survival of *Salmonella* after one week of storage (Table 4). The surviving *Salmonella* count was minimal at 22°C. At 4°C the mean count of SS was 33 MPN/cm (1.52 $\log_{10}$) and 3 MPN/cm (0.48 $\log_{10}$) at weeks one and two, respectively, compared with <0.9 MPN/cm (<−0.05 $\log_{10}$) for STM. None of the *Salmonella* strains survived after four-week storage under either refrigerated or ambient conditions. The overall trend in $\log_{10}$ reduction of the *Salmonella* count was similar for both strains.

Table 4: Survival on egg shells during storage at 4 and 22°C.

Time (weeks)	4°C ($\log_{10}$MPN/cm^2)[1]		22°C ($\log_{10}$MPN/cm^2)	
	SS[2]	STM[3]	SS	STM
0	3.89^{aA}	3.39^{abA}	3.55^{abA}	2.88^{bA}
1	1.52^{aB}	$<-0.05^{bB}$	0.56^{bB}	0.25^{bB}
2	0.48^{aC}	$<-0.05^{aB}$	0.10^{aB}	$<-0.05^{aB}$
4	$<-0.05^{aC}$	$<-0.05^{aB}$	$<-0.05^{aB}$	$<-0.05^{aB}$

Different lower case letters within rows are significantly different and different upper case letters within columns are significantly different ($P < 0.01$).
[1]Mean MPN/cm^2 of five eggs at each sampling time.
[2]SS: *S.* Sofia 1296a; [3]STM: *S.* Typhimurium PT135.

4. Discussion

As anticipated, all of the *Salmonella* isolates reached stationary phase rapidly in egg yolk when incubated at 37°C. The growth rate was not different between SS, RS, and the egg-related outbreak strain STM in any egg component, regardless of temperature. The nutrient-rich environment in the egg yolk allows the quick growth of SE [13] and this would not be expected to be different for other *Salmonella* serovars. Even at 15°C, stationary phase was reached after three days in the egg yolk and four days in the whole egg for all of the isolates in this study. This indicates that if *Salmonella* were to contaminate eggs and access the egg yolk, *Salmonella* numbers would be very high before the best before dates of eggs were reached, even with the eggs stored at the recommended AECL storage temperature of 15°C [11]. In a study by Cogan and others [14] *Salmonella* levels of >10^6 CFU/mL were used to indicate that SE and STM had been able to invade the egg yolk after inoculation in the albumen. These authors concluded that motility of the isolates was necessary for this to occur and that the presence of curli fimbriae permitted greater growth of the *Salmonella* [14]. Both SS and RS have the presence of curli fimbriae [15], although this is undetermined for STM. It is unknown whether or not the presence of fimbriae may have resulted in the varied growth rates in egg white in this study and is worthy of further investigation.

Musgrove et al. [16] looked at the growth of a two strain cocktail of STM DT104 at 4, 10, 20, 30, 37, and 42°C in pasteurized liquid whole egg and egg white, although the trials did not extend beyond 16 days. STM grew at all temperatures in whole egg except at 4°C and also grew in egg white from 20 to 37°C, but not at 4, 10, or 42°C [16]. The STM isolates in the current work, as well as the *Salmonella* Sofia, appeared to reach stationary phase more quickly at 37°C than the STM DT104 isolates (12–16 h versus 20 h, resp.), even with a lower starting inoculum. That study used an initial inoculum of 10^4 CFU/mL [16] which would be considered a high starting inoculum given that eggs naturally contaminated with *Salmonella* would generally have <20 CFU/egg [17]. If a lower starting inoculum was used, the time to reach stationary phase in egg yolk or a whole egg would increase. This would also increase the time before an infectious dose was reached. The infectious dose of *Salmonella* spp. is considered to be 10^2-10^3 CFU/mL [18]. However, it would only take the isolates in this study two days at 15°C and 20 h at 22°C to reach an infectious dose in a whole

egg if starting with a count of 20 CFU/egg. In order to prevent the growth of *Salmonella* in eggs, it is critical that eggs can be stored at temperatures that inhibit the growth of *Salmonella*, which is below 7°C for most salmonellae [19].

In the current study, attachment levels were always lower after 1 min compared to 20 min although only one combination was significant. The level of attachment of SS was significantly lower at 4°C compared to 42°C after 1 min. As the Code of Practice for shell eggs states that the wash water should be between 41 and 44°C, any *Salmonella* that may contaminate the wash water may attach at a higher level than if lower temperatures were used. This suggests that removal of gross faecal contamination as soon as possible after laying may assist in the reduction of the level of attached *Salmonella*. High levels of initial attachment have been noted for other organisms with rapid attachment within 1 min recorded for *Campylobacter* on stainless steel [20] and *Listeria* and *Pseudomonas* on raw potato tissue [21]. Temperature has also been shown to affect the level of attachment of *Campylobacter* and *Listeria* to stainless steel, with increasing levels of attachment noted with increasing temperature [20, 22], which was also seen when comparing SS on retail eggs at 4 and 42°C in contrast to other strains and conditions used in this study.

S. Sofia strain S1296a has been previously found to attach to five abiotic surfaces at significantly higher levels than *S.* Typhimurium strains, including the ATCC 14028 strain (RS) used in this study [23]. SS attached in significantly lower levels than RS after a period of 20 min attachment, but only on farm eggs. Properties of both the cell and the substrate are known to affect the attachment of bacteria. Surface properties such as surface roughness, surface free energy, and hydrophobicity may affect bacterial attachment and are reviewed by Goulter et al. [24]. STM, an egg outbreak strain, attached at similar levels to the SS, at 22°C with both strains attaching at significantly lower levels than RS. However at 4°C STM attached at levels similar to the other *S.* Typhimurium strain, RS. Attachment is a complex process and the limited number of strains and conditions used in this study along with the level of variation recorded make it difficult to ascertain precise temperatures for minimizing attachment. Further studies should examine both the surface of the egg and an increased number of strains.

One of the observations in the current investigation is the variability in recovery of *Salmonella* from egg surfaces. Other studies [25, 26] show similar variability in the recovery of *Salmonella* from egg surfaces and a rapid decline in viable numbers of SE adsorbed to egg surfaces. In the current study, egg surfaces inoculated with *Salmonella* were stored under refrigeration with high RH and ambient room temperature storage conditions that may occur in Australian consumers' households. The survival of SS after drying on the surface of eggs was similar to that of STM, with the only significant difference ($P < 0.01$) occurring after one week of storage where SS counts were higher at 4°C than at 22°C and higher than STM at both temperatures. In addition, SS was still at countable levels on egg shells after two-week storage, suggesting that SS may have an advantage in survival and particularly under refrigeration conditions. This is contrary to published literature which suggests that the greater survival [26] of *Salmonella* at 22°C rather than 4°C after seven days storage is likely related to high humidity under refrigerated conditions compared to ambient temperature. In Australia, SS is the dominant *Salmonella* serotype on chicken carcasses both before and after processing [27]. These results may indicate that SS can endure certain stresses that occur in the final, chilled stages of poultry processing better than other *Salmonella* serovars in Australia.

5. Conclusion

This study aimed to determine if the egg outbreak-related strain of *S.* Typhimurium (STM) grew in or attached and survived on egg shells in higher levels than a strain of *S.* Sofia (SS) (not associated with eggs) and a reference strain of *Salmonella* Typhimurium (RS). *Salmonella* egg outbreaks in Australia are associated with contamination on the egg, not due to organisms internalized during the formation of the egg; thus information on the attachment of a *Salmonella* outbreak strain on egg shells is new and valuable for understanding how to limit contamination of eggs with *Salmonella*. Differences in the growth of these strains in egg contents were not observed, with all of the isolates growing rapidly to stationary phase in egg yolk and whole egg, but not egg white. A first step in the contamination of eggs with *Salmonella* is the attachment of *Salmonella* to the egg shell. In this study, the egg outbreak-associated STM was not able to attach to eggs in significantly higher levels than the other strains and it is likely that factors other than attachment to egg surfaces contributed to the reasons that this particular serotype caused an outbreak associated with eggs. Furthermore, the SS and STM strains exhibited similar survival on the surface of the eggs and survival was minimal after two weeks and not detected after four weeks. Bacterial survival on egg surfaces decreased under storage at refrigerated temperature (4°C) associated with high humidity (>88% RH) more than under ambient conditions of temperature and humidity. Notwithstanding the need to maintain hygienic egg harvesting, washing and distribution handling practices, it is critical that eggs can be stored at temperatures less than 7°C, which is the accepted minimum temperature for growth of *Salmonella* in food. Existing industry practices of maintaining a retail product shelf-life of six weeks would be best undertaken by maintaining refrigeration conditions, desirably at 4°C as indicated in the current study, so as to limit the risk of survival of *Salmonella* and other microflora present on eggs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Molecular Epidemiology of Invasive Listeriosis due to *Listeria monocytogenes* in a Spanish Hospital over a Nine-Year Study Period, 2006–2014

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We investigated the pathogenicity, invasiveness, and genetic relatedness of 17 clinical *Listeria monocytogenes* stains isolated over a period of nine years (2006–2014). All isolates were phenotypically characterised and growth patterns were determined. The antimicrobial susceptibility of *L. monocytogenes* isolates was determined in E-tests. Invasion assays were performed with epithelial HeLa cells. Finally, *L. monocytogenes* isolates were subtyped by PFGE and MLST. All isolates had similar phenotypic characteristics (β-haemolysis and lecithinase activity), and three types of growth curve were observed. Bacterial recovery rates after invasion assays ranged from 0.09% to 7.26% (1.62 ± 0.46). MLST identified 11 sequence types (STs), and 14 PFGE profiles were obtained, indicating a high degree of genetic diversity. Genetic studies unequivocally revealed the occurrence of one outbreak of listeriosis in humans that had not previously been reported. This outbreak occurred in October 2009 and affected three patients from neighbouring towns. In conclusion, the molecular epidemiological analysis clearly revealed a cluster (three human cases, all ST1) of not previously reported listeriosis cases in northwestern Spain. Our findings indicate that molecular subtyping, in combination with epidemiological case analysis, is essential and should be implemented in routine diagnosis, to improve the tracing of the sources of outbreaks.

1. Introduction

Listeria monocytogenes is an emerging foodborne pathogen capable of infecting animals and humans. It is the leading cause of death in reported cases of food poisoning [1]. The groups at highest risk of listeriosis are pregnant women, in whom this infection may cause late miscarriage or stillbirth, neonates, immunocompromised individuals, and the elderly, in whom it causes mostly septicaemia and meningoencephalitis [2, 3]. The incidence of listeriosis has recently been reported to be higher among the elderly than in other groups [4, 5]. The case-fatality rate is still increasing (20–30%) worldwide, despite antibiotic treatment [6, 7]. Noninvasive listeriosis is often associated with febrile gastroenteritis and sometimes with cutaneous forms, as observed in veterinary surgeons coming into direct contact with aborted foetuses from livestock [8, 9]. The disease is usually vertically transmitted during pregnancy or acquired by the consumption of contaminated food, particularly fresh and ready-to-eat products that are not heated before consumption [10, 11].

A European Food Safety Authority (EFSA) baseline study focusing on ready-to-eat (RTE) food indicated that smoked and marinated fish products carried the highest risk of *L. monocytogenes* contamination [1]. In the US, poultry meat was found to be responsible for most (63%) fatal cases of listeriosis [12]. A recent study showed that foodborne outbreaks in hospitalised patients on immunosuppression treatments were linked to hospital food (e.g., sandwiches and celery) [5].

L. monocytogenes consists of four discrete evolutionary lineages (I–IV) and 13 serotypes [13, 14]. Historically, diverse molecular typing methods, including PFGE, multilocus enzyme electrophoresis, and ribotyping, have been used to study the genetic diversity of the isolates involved in international outbreaks. Thus, epidemic clones (ECs) involved in geographically and temporally distant outbreaks or in large, single outbreaks have been defined [15]. In the last decade, ECs have been redefined on the basis of multi-virulence-locus sequence typing (MVLST), which is based on the analysis of six to eight genes [16]. Multilocus sequence typing (MLST), which is based on the analysis of seven housekeeping genes [17], has also been used for the definition of clonal groups. Sequence types (ST) are defined as a unique combination of MLST allele designations used in the MLST scheme, and clonal complexes (CC) are defined as groups of STs differing by only one housekeeping gene from other members of the group. An analysis of *L. monocytogenes* isolates from five continents by MLST demonstrated the existence of globally successful genetic groups [18]. Seven "epidemic clones" (ECs) have been defined by MVLST, each descended from a common ancestor with a similar temporal and spatial virulence profile [16, 17, 19, 20]. ECI, corresponding to the CC1 identified by MLST, and ECIV (CC2) appear to be cosmopolitan clones involved in many outbreaks [16]. *L. monocytogenes* sequence type (ST) 6 (ECII) has been implicated in human meningitis with a fatal outcome [21]. *L. monocytogenes* serogroups most frequently associated with clinical cases are serotype 4b, followed by 1/2b (genetic lineages I and III), and 1/2a (genetic lineage II) [14]. *L. monocytogenes* serotype 1/2a is increasingly being isolated from cases of invasive listeriosis in Italy and Switzerland [21–24]. A link between isolates obtained from patients and isolates obtained from smoked fish has been reported in Scandinavian countries (Sweden, Norway, and Finland) and in eastern Spain [25–29]. Furthermore, actual outbreaks of listeriosis have been linked to *L. monocytogenes* serotype 1/2a and seem to be particularly prevalent in cheese processing plants [10, 30–33].

The incidence of listeriosis in Spain has increased steadily over the last decade. There were a reported 0.56 cases per 100,000 inhabitants from 2001 to 2007 [34]. Martínez et al. [35] reported 0.67 invasive listeriosis cases per 100,000 inhabitants in Valencia during the 2008–2010 period. In 2012, the notification rate for listeriosis cases in Spain was the second highest of any member state of the EU (0.93, versus an EU-wide rate of 0.41 per 100,000 inhabitants) [6].

Mortality rates are high for invasive listeriosis, justifying the use of combinations of molecular subtyping tools for the identification of clusters associated with outbreaks, tracing the source of the outbreak, and preventing further transmission. These methods were therefore combined in a retrospective study focusing on invasive listeriosis cases in León (2006–2014) and involving *in vitro* virulence testing.

2. Materials and Methods

2.1. Case Definition. All patients suffering from meningitis, bacteraemia, or infection during pregnancy were considered as potential cases of listeriosis. The infection was confirmed by the isolation of *L. monocytogenes* from a normally sterile site.

2.2. Description of the Hospital. The study has been carried out in the "*Complejo Asistencial Universitario de León*" (CAULE), a facility with about 800 beds located in the province of León in Northwest Spain. It serves an urban population of over 130,000 inhabitants and the total population of the metropolitan area has been estimated at over 490,000.

2.3. Clinical Cases. In total, there were 17 clinical cases of listeriosis at the CAULE from 2006 to 2014. These cases occurred in one premature newborn and 11 male and five female patients, aged from 31 to 89 years. In total, 11 *L. monocytogenes* isolates were recovered from blood cultures, and six were recovered from cerebrospinal fluid (CSF). In some patients *L. monocytogenes* isolates were recovered from both blood cultures and CSF ($n = 2$) or from peritoneal fluid (PF; $n = 1$) or synovial joint fluid (JF; $n = 1$) (Table 1).

2.4. Isolation and Confirmation of L. monocytogenes. Clinical isolates of *L. monocytogenes* were streaked onto two selective chromogenic agar plates: ALOA (Agar Listeria Ottaviani & Agosti) medium (CHEMUNEX, Bruz Cedex, France) and RAPID L. mono agar (Bio-Rad Laboratories, Inc., Hercules, Ca, US). The bacteria were subjected to Gram staining and catalase and Christie Atkins Munch-Petersen (CAMP) tests. The collection strain *S. aureus* CECT 828 was used in the CAMP test, as recommended, to enhance *L. monocytogenes* haemolysis.

Each of the *L. monocytogenes* isolates was confirmed biochemically (API Coryne V.2.0; bioMérieux, Marcy l'Etoile, France) and by real-time PCR methods for differentiating between *Listeria* species [36–39]. The *L. innocua* CECT910, *L. monocytogenes* ITA1315, and *L. ivanovii* ATCC19119 reference strains served as positive controls in the PCR assays.

2.5. L. monocytogenes Growth Curves. Growth curves were determined by culture in brain heart infusion (BHI, Oxoid, Hampshire, UK), with the measurement of optical density at 600 nm in a Lambda 35 UV/VIS Spectrometer (PerkinElmer, Massachusetts, USA) over a 24-hour period of incubation at 37°C, with shaking at 180 rpm.

2.6. Antimicrobial Susceptibility Testing. The susceptibility of 17 clinical *L. monocytogenes* isolates to 16 antimicrobial agents was determined by E-tests on Mueller-Hinton agar supplemented with 5% sheep's blood, incubated under an atmosphere of ambient air at 35°C, with reading of the plates

Table 1: Clinical cases of invasive *Listeria monocytogenes* infections in a Spanish hospital over a nine-year study period, 2006–2014.

Sample	Sex	Age	Date	Source	Clinical diagnosis	Antibiotic treatment	Province	PFGE pulsotype	MLST ST
Genetic lineages I and III									
1	M	60	Jun. 06	CSF	Meningoencephalitis	Ampicillin	León	H	1
8	M	44	Oct. 09	CSF	Meningoencephalitis	Ampicillin/gentamicin	León	D	1
9[†]	F	68	Oct. 09	Blood culture/CSF	Meningoencephalitis/sepsis	Ampicillin/gentamicin/vancomycin	León	D	1
10	M	NB	Oct. 09	Blood culture	Sepsis	Ampicillin/gentamicin	Zamora	D	1
7	M	76	Feb. 09	Blood culture/JF	Arthritis/bacteraemia	Ampicillin/gentamicin	León	G	2
6	M	83	Jan. 09	Blood culture	Bacteraemia	Ampicillin/gentamicin	León	E	3
11	M	50	Feb. 10	Blood culture	Sepsis	Ampicillin/gentamicin	Asturias	A	3
15	M	82	Jul. 13	CSF	Meningoencephalitis	Ampicillin/gentamicin	León	L	4
13	F	89	Feb. 12	Blood culture	Sepsis	Ampicillin/gentamicin	León	B	87
4[†*]	F	31	Apr. 07	Blood culture	Fever in pregnant woman	Amoxicillin clavulanate	León	F	389
Genetic lineage II									
5	M	50	Dec. 08	CSF	Meningoencephalitis	Ampicillin/gentamicin	León	K	9
2	M	84	Sep. 06	Blood culture/PF	Peritonitis/sepsis	Ampicillin/vancomycin	León	I	16
3	M	40	Feb. 07	CSF	Meningoencephalitis	Ampicillin/gentamicin	León	I	16
12	M	65	Apr. 10	Blood culture	Sepsis	Ampicillin/gentamicin	León	J	16
14	F	82	Jun. 12	Blood culture	Sepsis	Ampicillin/gentamicin	León	C	399
16	M	59	Feb. 14	CSF	Meningoencephalitis	Ampicillin/gentamicin	León	M	7
17	F	63	Feb. 14	Blood culture/CSF	Meningoencephalitis/sepsis	Ampicillin/gentamicin	León	N	8

[*]Foetal death. Full recovery of the mother; [†]Deceased.

PFGE, pulsed-field gel electrophoresis; MLST, multilocus sequence typing; NB, premature newborn; CSF, cerebrospinal fluid: JF, joint fluid; PF, peritoneal fluid.

after 20–24 h. Susceptibility to antibiotics was interpreted applying the recommendations of the EUCAST for the antimicrobial susceptibility of *L. monocytogenes* [40]. The following antibiotics were tested: benzylpenicillin, ampicillin, imipenem, meropenem, erythromycin, clindamycin, gentamicin, vancomycin, daptomycin, linezolid, ciprofloxacin, moxifloxacin, tetracycline, tigecycline, rifampin, and cotrimoxazole.

2.7. Invasion Assays. We assessed the invasiveness of 17 clinical *L. monocytogenes* isolates in an epithelial HeLa cell culture assay, as previously described [41]. A well characterised clinical *L. monocytogenes* serovar 4b strain (P14) and its isogenic *prf*A gene deletion mutant (Δ*prf*A) were included in the assay as controls. HeLa ATCC CCL-2 cells were maintained at 37°C, under an atmosphere containing 5% CO_2. The invasiveness of each *L. monocytogenes* isolate was analysed in quadruplicate (2 independent invasion assays, with each isolate analysed in duplicate in each assay). Between passages 1 and 14, cell lines were maintained in Eagle's minimum essential medium (MEM; Gibco, San Diego, United States) supplemented with 2 mM L-glutamine, 10% foetal bovine serum, and 1% nonessential amino acids. Bacteria were resuspended in plain Eagle's minimum essential medium and used to infect HeLa cells at a multiplicity of infection (MOI) of 20 : 1, with the exception of the Δ*prf*A mutant strain of *L. monocytogenes,* for which we used a MOI of 200 : 1.

2.8. Molecular Epidemiological Analysis. L. monocytogenes serogroups were defined according to a multiplex PCR targeting the specific target genes *lmo*0737, *lmo*1118, *ORF*2819, *ORF*2110, and *Listeria* spp.-specific *prs* published by Doumith et al. [42] and amended by Leclercq et al. [43] for PCR IVb-VI. *L. monocytogenes* clinical isolates were genomically characterised by pulsed-field gel electrophoresis (PFGE) with the restriction enzymes *Apa*I and *Asc*I, according to the standardised international protocol of PulseNet [44]. We analysed PFGE profiles with Bionumerics v.6.6 software (Applied-Maths NV, Sint-Martens-Latem, Belgium), to describe the genetic relationships between isolates. Dendrograms were constructed with the Dice similarity coefficient and the unweighted pair group mathematical average (UPGMA) clustering algorithm. Tolerance and optimisation values were set to 1.5%, in accordance with the recommendations of Martin et al. [45]. Simpson's index of diversity, which measures the probability of two unrelated strains sampled from the test population being placed in different typing groups [46], was calculated to compare the discriminative power of PFGE, via a website comparing partitions [47]. Fingerprints were interpreted according to the recommendations for foodborne pathogens [48]. Multilocus sequence typing (MLST) was performed as described by Ragon et al. [17]. Allele types were assigned for seven housekeeping loci, *abc*Z (ABC transporter), *bgl*A (beta glucosidase), *cat* (catalase), *dap*E (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), and *lhk*A (histidine kinase), and the resulting sequence types (STs) were determined and compared, with the Institute Pasteur *Listeria monocytogenes* MLST database [49]. Sequence types (STs) were defined as a unique combination of MLST allele designations used in the MLST scheme, and clonal complexes (CC) were defined as groups of STs differing by only one housekeeping gene from other members of the group [17]. An allelic profile-based comparison, based on the use of a minimum spanning tree (MST) and the Pasteur Institute online tool, was performed to define the relationships between strains at the microevolutionary level.

2.9. Statistical Analysis. Statistical two-way analysis of variance (ANOVA) was used to evaluate differences in invasive capacity between isolates, based on the 95% confidence interval and Bonferroni multiple comparison tests to assess the differences in greater depth (GraphPad Prism v.5.0). The threshold *P* value for this test was set at 0.05.

3. Results

3.1. Patient Outcomes. The outcome was favourable after antibiotic treatment in 15 of the 17 patients. Progression was observed in case 4, a pregnant woman whose foetus died as a result of the infection. Patient 9 died after antibiotic treatment failure. Further information is provided in Table 1.

3.2. L. monocytogenes Isolation, Confirmation, and Growth Curves. All 17 clinical isolates were confirmed to be *L. monocytogenes* by phenotypic and genetic methods. β-haemolytic and lecithinase activities, at 24, 48, and 72 hours, were similar in all the clinical isolates. As expected, these activities were stronger in *L. ivanovii* ATCC19119, and *L. innocua* CECT910 displayed no activity. The isolates recovered from clinical cases 6 and 7 had specific growth patterns (GPs), reaching the exponential growth phase later than the other isolates (exponential growth phase from 5 to 12 hours and from 13 to 23 hours of incubation, resp.). The exponential phase in the other *L. monocytogenes* isolates began after about three hours and continued until about 6.5 hours of incubation. Representative growth curves for the isolates are shown in Figure 1.

3.3. Antimicrobial Susceptibility Testing. The antimicrobial susceptibility data for the 17 isolates tested are presented in Table 2. In the E-test method, all our isolates were found to be susceptible to benzylpenicillin, ampicillin, erythromycin, and cotrimoxazole. In 10 isolates, the MIC of meropenem was 0.38 to 0.75 mg/L. These isolates may be considered resistant, according to the strict criterion of the EUCAST susceptibility breakpoint. No susceptibility breakpoints have been identified for the other 11 antibiotics by EUCAST, and the MICs of these antibiotics ranged between 0.125 and 4 mg/L.

3.4. Invasion Assays. All clinical isolates of *L. monocytogenes* were tested in invasion assays with HeLa epithelial cells. The actual mean MOI used for cell infection with the clinical isolates was 17 : 1 (standard error: 0.83). The recovery rates for clinical isolates ranged from 0.09% to 7.26% (median: 1.62, standard error: 0.46). As expected, the recovery rate for

Table 2: Antimicrobial susceptibility of 17 *L. monocytogenes* isolates in the E-test.

Antibiotic	MIC (mg/L)			Susceptibility breakpoint (mg/L)[a]	% susceptibility
	Range	50%	90%		
Benzylpenicillin	0.064–1	0.25	0.75	≤1	100
Ampicillin	0.064–1	0.25	0.75	≤1	100
Imipenem	0.125–0.19	0.19	0.19	—	—
Meropenem	0.19–0.75	0.38	0.38	≤0.25	58.8
Erythromycin	0.125–0.38	0.25	0.38	≤1	100
Clindamycin	0.25–8	2	4	—	—
Gentamicin	0.094–1	0.25	0.5	—	—
Vancomycin	0.75–1.5	1.5	1.5	—	—
Daptomycin	0.75–1.5	1	1.5	—	—
Linezolid	1-2	2	2	—	—
Ciprofloxacin	0.5–2	1	1.5	—	—
Moxifloxacin	0.19–0.5	0.38	0.5	—	—
Tetracycline	0.094–1.5	1	1.5	—	—
Tigecycline	0.094–1.5	0.125	0.25	—	—
Rifampin	0.023–0.19	0.094	0.125	—	—
Cotrimoxazole	0.008–0.023	0.012	0.019	≤0.06	100

[a]According to EUCAST antimicrobial susceptibility breakpoints for *L. monocytogenes*.

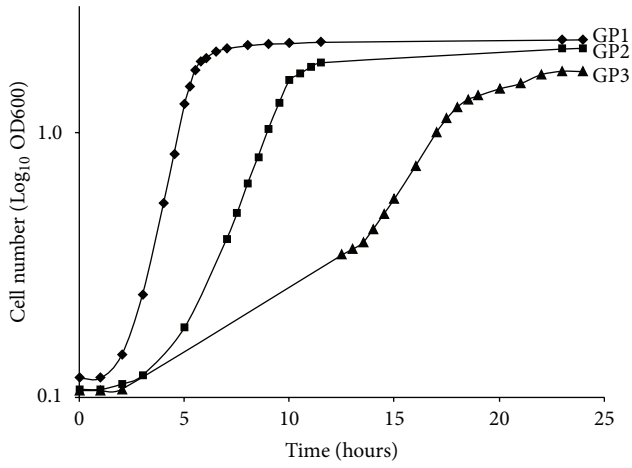


Figure 1: Growth patterns of 17 clinical isolates of *Listeria monocytogenes* causing invasive infections at the "Complejo Asistencial Universitario de León" from 2006 to 2014. Two of the isolates had growth patterns different from that of all the other isolates: isolate 6 (GP2) and isolate 7 (GP3), respectively.

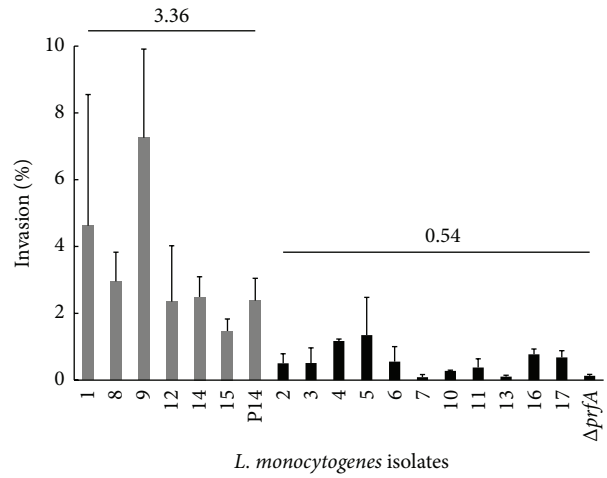
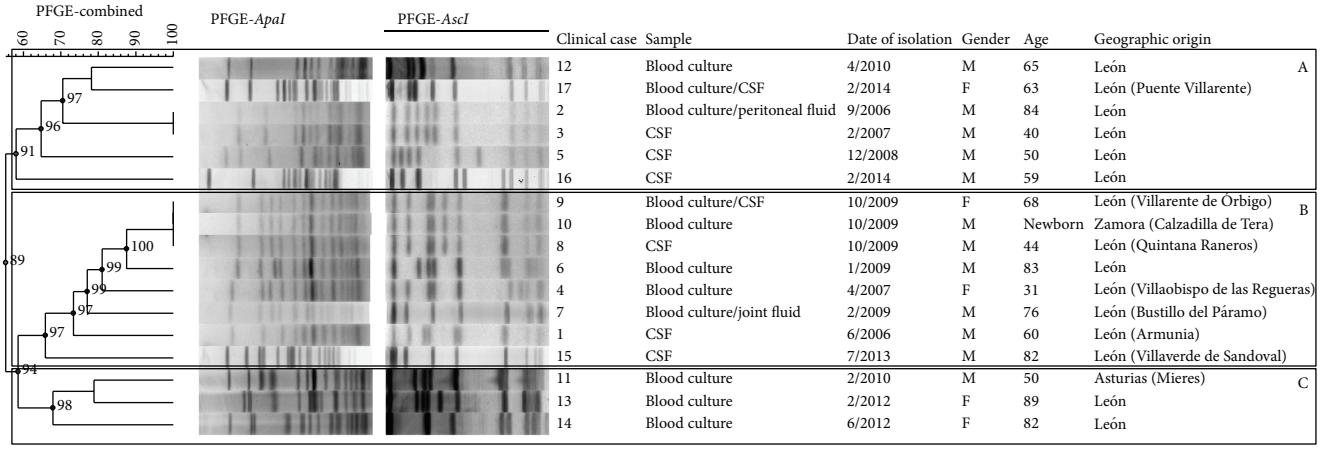


Figure 2: Invasion assays for the 17 clinical isolates of *Listeria monocytogenes* in HeLa epithelial cells. The mean number of internalised bacteria as a percentage of the initial inoculum is shown on the *y*-axis. The error bars show the standard error of two independent experiments, each performed in duplicate. The wild-type *L. monocytogenes* P14 and noninvasive *L. monocytogenes* Δ*prfA* strains were included, to assess the reproducibility of the experiments.

the noninvasive Δ*prf*A mutant strain was very low (0.13%, standard error: 0.04). Invasiveness was between 0.04 and 3.1 times higher than that of the *L. monocytogenes* serovar 4b clinical control strain (P14) (Figure 2). The isolates clustered into two significantly different groups: those with a high invasion rate similar to that of the wild-type strain P14 (3.36 ± 0.74) (isolates from clinical cases 1, 8, 9, 12, 14, and 15) and isolates with a low invasion rate, similar to that of the isotypic Δ*prf*A strain (0.54 ± 0.12) (isolates from clinical cases 2, 3, 4, 5, 6, 7, 10, 11, 13, 16, and 17 (Figure 2)).

3.5. Molecular Epidemiological Analysis. Genetic characterisation by PFGE with the restriction enzyme *Apa*I revealed 13 different pulsotypes, and characterisation with *Asc*I discriminated between 14 genotypes (Simpson's index of diversity values of 0.949 and 0.971, resp.). Fingerprinting revealed the presence of 8–20 DNA fragments between about 40 and 560 kb in size. PFGE analyses combining the results



Clinical case	Sample	Date of isolation	Gender	Age	Geographic origin	
12	Blood culture	4/2010	M	65	León	A
17	Blood culture/CSF	2/2014	F	63	León (Puente Villarente)	
2	Blood culture/peritoneal fluid	9/2006	M	84	León	
3	CSF	2/2007	M	40	León	
5	CSF	12/2008	M	50	León	
16	CSF	2/2014	M	59	León	
9	Blood culture/CSF	10/2009	F	68	León (Villarente de Órbigo)	B
10	Blood culture	10/2009	M	Newborn	Zamora (Calzadilla de Tera)	
8	CSF	10/2009	M	44	León (Quintana Raneros)	
6	Blood culture	1/2009	M	83	León	
4	Blood culture	4/2007	F	31	León (Villaobispo de las Regueras)	
7	Blood culture/joint fluid	2/2009	M	76	León (Bustillo del Páramo)	
1	CSF	6/2006	M	60	León (Armunia)	
15	CSF	7/2013	M	82	León (Villaverde de Sandoval)	
11	Blood culture	2/2010	M	50	Asturias (Mieres)	C
13	Blood culture	2/2012	F	89	León	
14	Blood culture	6/2012	F	82	León	

Figure 3: Genetic relationships between 17 clinical isolates of *Listeria monocytogenes*, based upon comparison of pulsed-field gel electrophoresis profiles obtained with the restriction enzymes *Apa*I and *Asc*I. The dendrogram was produced with a Dice similarity coefficient matrix, using the unweighted pair group method with arithmetic mean (UPGMA). Tolerance and optimisation values were set to 1.5%. Clusters are arbitrarily designated A to C. Scale bar indicates similarity values.

obtained with both restriction enzymes identified 14 unique pulsotypes, resulting in a Simpson's index of diversity of 0.971. The clinical isolates displayed 55% similarity and formed three clusters, arbitrarily designated A to C. Cluster A contained six isolates recovered from 2006 to 2014 (58% similarity). Cluster B contained eight isolates recovered from 2006 to 2013 (66% similarity). Cluster C consisted of three isolates (68% similarity) collected from 2010 to 2012. Interestingly, the isolates from clinical cases 8, 9, and 10 on one hand and those from cases 2 and 3 on the other hand had indistinguishable pulsotypes (pulsotypes D and I, resp.). The genetic relationships between *L. monocytogenes* isolates, based on the combined PFGE-genetic profiles obtained with the restriction enzymes *Apa*I and *Asc*I, are shown in Figure 3.

MLST analysis of 17 *L. monocytogenes* isolates from clinical cases of listeriosis identified 11 STs (Simpson's index of diversity: 0.926). The oldest and globally most prevalent epidemic clones (ST1, ST2, ST3, ST4, ST7, ST8, and ST9) were represented among the STs of the clinical cases observed in the Spanish regions of León, Asturias, and Zamora in 2006–2014 (Table 1; Figures 4(a) and 4(b)). Ten isolates (58.8%) were assigned to genetic lineage I or III. Four of these isolates were ST1 isolates (3 with PFGE profile D and 1 with PFGE profile H) recovered from human patients from the neighbouring provinces of León and Zamora during 2006 and 2009. ST3 isolates were obtained from two patients from the provinces of León and Asturias in 2009 and 2010, respectively. These cases were not related and the isolates concerned had different PGFE profiles (A and E). Furthermore, ST2, ST4, ST87, and ST389 were sporadically observed, in one isolate each. The *L. monocytogenes* isolates from genetic lineages II ($n = 7$; 41.2%) were more evenly distributed in the MST. The most common allelic profile was ST16 ($n = 3$; 17.6%). ST7, ST8, ST9, and ST399 were observed sporadically, in one isolate each (Table 1).

4. Discussion

Most of the reported cases of listeriosis occur in high-income countries, this infection being largely underreported in developing countries. Hospitalisation records show listeriosis to be the third most costly zoonotic disease in the US [34, 50]. Patients often suffer from comorbid diseases and are immunocompromised, and long-term antibiotic treatment with ampicillin, amoxicillin, and gentamicin may be required. Some *L. monocytogenes* strains can survive treatment with cephalosporin or erythromycin [51]. Livestock and processed foods seem to serve as a source of antibiotic resistance. Some authors have reported increases in the frequency of multidrug-resistant strains (e.g., resistant to amoxicillin-clavulanate and chloramphenicol) [25, 52]. No resistance to benzylpenicillin, ampicillin, erythromycin, or cotrimoxazole was found in our isolates. Remarkably, 10 isolates were classified as resistant to meropenem according to the EUCAST breakpoint for this antibiotic, potentially discouraging its use to treat meningitis. All patients were treated with betalactams (ampicillin or amoxicillin), mostly in combination with gentamicin. Treatment outcome was favourable in 15 patients (88.2%). Mortality is known to be high in patients with invasive listeriosis, severe underlying diseases, meningoencephalitis, and inadequate antimicrobial treatment. The early administration of antibiotics, such as ampicillin or cotrimoxazole, which have rapid bactericidal activity against the pathogen, is essential for cure.

The 17 *L. monocytogenes* clinical isolates included in this study had phenotypic properties consistent with full virulence: β-haemolysis mediated by the product of the listeriolysin gene (*hly*) and lecithinase activity due to the presence of phospholipases (*plcA* and *plcB*). Some differences in growth patterns and invasiveness were observed. Growth pattern (GP) 1 was observed in all but two of the *L. monocytogenes* isolates (87.5% of all isolates). The *L. monocytogenes*

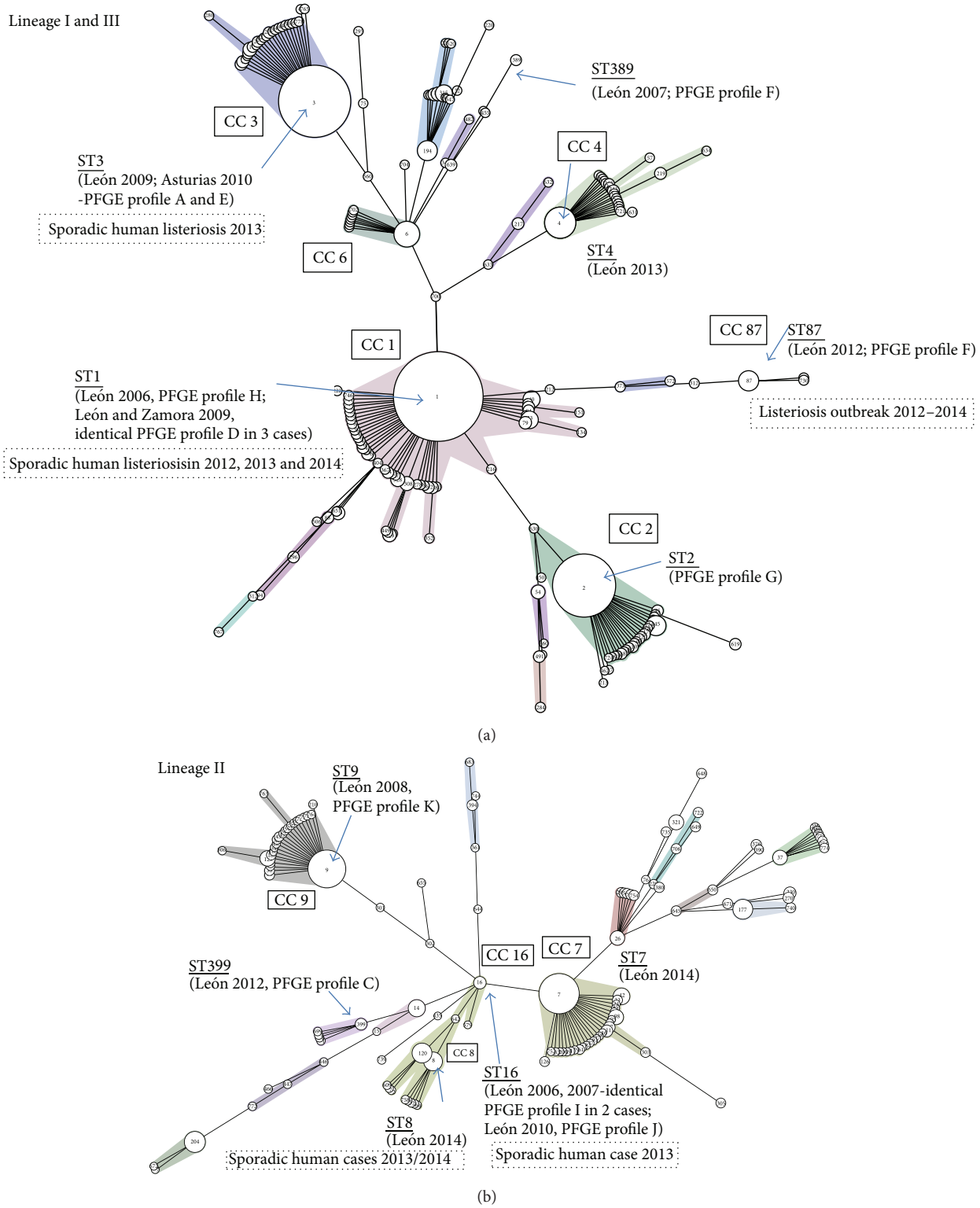


Figure 4: Multilocus sequence typing of 17 *Listeria monocytogenes* isolates from sporadic cases of human listeriosis in Spain during the 2006–2014 period. The sequence types were clustered according to the sequence of the *abcz* housekeeping gene, using a minimum spanning tree (MST) tool available from the Pasteur Institute MLST database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/). The STs from genetic lineages I and III (a) and genetic lineage II (b) found in this study are underlined. Sporadic listeriosis cases and outbreaks in Spain (2012–2014) listed in the Pasteur Institute MLST database are shown in boxes outlined with dotted lines. *L. monocytogenes* sample origins and PFGE profiles are included in each MST. The coloured zones surrounding groups of STs indicate clonal complexes (CC) differing by only one gene from other members of the group.

isolates from cases 6 and 7 (STs 3 and 2) reached the exponential growth phase later than the other isolates (Figure 1). In cell culture assays, invasiveness varied considerably between clinical isolates, with recovery rates ranging from 0.09% to 7.26% (mean 1.62%, standard error 0.46) (Figure 2). The recovery rates for five clinical isolates were reported to range from 4.3% to 30% in a previous study on Vero cells [53]. *L. monocytogenes* isolates 9, 1, and 8 (all ST1) had the highest levels of virulence in cell culture assays *in vitro*, as shown by their invasiveness in HeLa cells (invasiveness up to three times that of the wild-type P14 strain). This finding is consistent with those of most previous studies, reporting that lineage I (4b) strains seem to be more virulent and better able to withstand the adverse conditions present in the stomach of the host [19]. ANOVA revealed the existence of two groups of isolates. Group I consisted of six clinical isolates (35.3%) and the wild-type P14 strain, all with significant levels of invasiveness. By contrast, group II contained 11 isolates (64.7%) with nonsignificant levels of invasiveness, similar to that of the isogenic control strain Δ*prf* A.

In our study of invasive listeriosis cases, the percentage of lineages I and III isolates of *L. monocytogenes* was similar to that of lineage II isolates (58.8% versus 41.2%, resp.). The isolates responsible for the death of a 68-year-old woman and a foetus were assigned to ST1 and ST389, respectively (lineage I/III, clinical cases 9 and 4). Overall, PFGE identified 14 genotypes and MLST identified 11 genotypes among the 17 *L. monocytogenes* clinical isolates, yielding values of Simpson's index of diversity of 0.971 and 0.926, respectively. This indicates a high level of genomic diversity among the clinical isolates (only 55% similarity on PFGE) despite their recovery at the same hospital, consistent with the findings of previous molecular epidemiology studies.

An analysis of the genomic relationships between isolates unequivocally revealed the occurrence of one previously unreported outbreak of listeriosis in humans. This outbreak occurred in October 2009 and affected three patients living in neighbouring towns (clinical cases 8, 9, and 10: pulsotype D, ST1). Isolate 10 had a significantly lower invasion capacity than isolates 8 and 9 (Figure 2), but this difference may simply reflect the method used, with invasion capacity being evaluated *in vitro*. *L. monocytogenes* ST1 isolates from Spanish patients are also present in the Pasteur Institute MLST database (2012–2014; Figure 4(a)). Moreover, an epidemiological connection was identified between two isolates from 2006 and 2007, both of which belonged to ST16 and had a PFGE profile I. Data for *L. monocytogenes* ST16 and ST8 isolates from sporadic clinical cases of human listeriosis are also available from the MLST database (Figure 4(b)). Many previous molecular epidemiology studies have detected otherwise unreported outbreaks of listeriosis. In a recent Spanish study, a large proportion of the clinical isolates had indistinguishable pulsotypes, suggesting the possible occurrence of listeriosis outbreaks related to international foodborne outbreaks. Most of these isolates were assigned to ECI (4b; CC1; 46.2%) and ECIII (1/2a; CC11; 33.3%) [54]. Thus, molecular epidemiology studies of *L. monocytogenes* can help to identify and trace the sources of outbreaks that would otherwise pass unnoticed.

Interestingly, this is the second time that ST87 has been linked to human disease (it was isolated in León in February 2012). Pérez-Trallero et al. [55] recently reported the occurrence of two outbreaks affecting 15 people and caused by ST87 strains in Guipúzcoa (northern Spain) in 2013 and 2014. Our study demonstrates that ST87 was already circulating in the Spanish clinical environment before the outbreak in Northern Spain. It would be interesting to follow the dissemination of this clone to assess its potential emergence.

In conclusion, this retrospective study focused on invasive *L. monocytogenes* infections in a Spanish healthcare institution over a nine-year study period. Molecular epidemiology studies clearly revealed the occurrence of a previously unreported outbreak of listeriosis in Northwest Spain. Our findings, along with those of previous studies [54–57], indicate that molecular epidemiology studies can help to identify and trace the source of the outbreaks that might otherwise pass unnoticed. Better centralised collection and subtyping of clinical isolates of *L. monocytogenes* would improve listeriosis monitoring, making it possible to trace the sources of Spanish outbreaks and to prevent cross-border outbreaks.

Abbreviations

CAULE: Complejo Asistencial Universitario de León
BHI: Brain heart infusion
MOI: Multiplicity of infection
MLST: Multilocus sequence typing
ST: Sequence type
MST: Minimum spanning tree
CC: Clonal complex
PFGE: Pulsed-field gel electrophoresis
GP: Growth pattern.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Comparison of Primary Models to Predict Microbial Growth by the Plate Count and Absorbance Methods

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The selection of a primary model to describe microbial growth in predictive food microbiology often appears to be subjective. The objective of this research was to check the performance of different mathematical models in predicting growth parameters, both by absorbance and plate count methods. For this purpose, growth curves of three different microorganisms (*Bacillus cereus, Listeria monocytogenes*, and *Escherichia coli*) grown under the same conditions, but with different initial concentrations each, were analysed. When measuring the microbial growth of each microorganism by optical density, almost all models provided quite high goodness of fit ($r^2 > 0.93$) for all growth curves. The growth rate remained approximately constant for all growth curves of each microorganism, when considering one growth model, but differences were found among models. Three-phase linear model provided the lowest variation for growth rate values for all three microorganisms. Baranyi model gave a variation marginally higher, despite a much better overall fitting. When measuring the microbial growth by plate count, similar results were obtained. These results provide insight into predictive microbiology and will help food microbiologists and researchers to choose the proper primary growth predictive model.

1. Introduction

Predictive microbiology enables, through the use of mathematical models, estimating the behaviour of microorganisms under certain circumstances [1], based upon the premise that the responses of microorganisms to environmental factors are reproducible. The ability to predict both the growth of microorganisms, as affected by different environmental factors, and the survival of microorganisms as a result of preservative treatments is an important tool for evaluating the safety and shelf life of food products.

Before predictive microbiology can be applied to the food industry, mathematical models that adequately describe microbial behaviour are needed. There are a number of sigmoid equations and several models that have been used as growth functions. They all differ in "ease of use" and number of parameters in the equation. Some authors have compared the behaviour of different growth models, from different viewpoints, including mathematical measures of goodness of fit [2] and/or other statistical criteria [3–5]. The usual measures of goodness of fit for model comparison in previous studies were done by calculating the bias (B_f) and accuracy (A_f) indices as provided by Ross [6], the coefficient of determination (r^2), the residual mean square error (RMSE), or the F-test. Other authors [7, 8] have focussed on direct

comparisons of particular growth parameters as predicted by various models.

These studies have reached different conclusions. Hence, there is significant disagreement in literature on which is the best-fitting model for predictive microbiology. The selection of a model in predictive food microbiology often appears to be subjective. Based on reports in the literature, Gompertz, Baranyi, Richards, logistic, and three-phase linear models are the most widely used [5, 9–11].

The growth curve has been mostly expressed in terms of microbial numbers (concentration of colony forming units), but also in terms of optical density as an indirect measurement. The measurement of absorbance is a rapid, nondestructive, inexpensive, and relatively easy-to-automate method to monitor bacterial growth, as compared to many other techniques and particularly when compared to classical viable count methods. When modelling optical density growth curves, the fitted parameters are different from the population growth parameters derived from viable counts. The rate of increase of the optical density does not express the maximum specific growth rate and the detection time is not equivalent to the lag time, unless the initial inoculum is greater than the detection limit. In spite of the limitations related to detection thresholds, correlation with the parameters derived from viable count growth curves, and inability to model growth in turbid liquid foods and in solid food matrixes, numerous techniques and mathematical growth models have been used in recent years for estimation of growth rates and lag times from absorbance data [5, 7, 8, 12–14]. In the opinion of Dalgaard and Koutsoumanis [7], absorbance techniques should be limited to conditions where high cell densities are reached, such as those resembling the growth of spoilage bacteria in foods. Even assuming the limitations of absorbance to build growth curves, it may be useful, if not to obtain very precise growth kinetic parameters, at least to compare the growth of different cultures or of the same cultures but in different conditions.

Work modelling the behaviour of bacteria in foods has shown that the lag phase is more difficult to predict than is the specific growth rate [15], mainly because of the influence in lag time of the physiological state of individual bacterial cells and, to a minor extent, of the inoculum size. The physiological state of the cells is affected by their previous growth environment and by exposure to stress conditions, which can extend the lag time considerably and also increase individual cell lag time variability [16–18]. However, microorganisms with a similar precultural history exposed to the same favourable growth conditions should be in a similar optimum physiological state and, thus, its effect on lag time variability is negligible [19]. Regarding the inoculum size, Baranyi and Pin [20] showed that as the cell number in the inoculum decreases, the population lag increases by an amount that depends on the distribution of individual lag times and the maximum specific growth rate. Augustin et al. [21] showed that the inoculum level effect can be explained by an increasing variability in individual cell lag time when stress factors become more stringent, and Baranyi and Pin [20] found that, under optimum growth conditions, this effect would only be expected at inoculum levels below about 10^2-10^3 cells, because the impact of variability among a small population of cells can become more important on lag time [2, 22].

Hence, a sufficiently large population of microorganisms exposed to exactly the same favourable growth conditions and with a similar precultural history behave in a similar way; that is, they should show the same growth parameters, growth rate, and lag phase duration.

The objective of this research was to check the performance of different mathematical models in predicting growth parameters, by both absorbance and plate count methods. For this purpose, growth curves of three different microorganisms (*Bacillus cereus, Listeria monocytogenes,* and *Escherichia coli*) grown, each species, under the same conditions, but with different initial concentrations, were analysed.

2. Materials and Methods

2.1. Microorganisms. *Bacillus cereus, Listeria monocytogenes,* and *Escherichia coli* were chosen as representative microorganisms for spore-forming, Gram-positive, and Gram-negative bacteria, respectively.

B. cereus INRA-AVTZ415 was kindly provided by the Institut National de la Recherche Agronomique (INRA, Avignon, France). *L. monocytogenes* and *E. coli* type strains (CECT 4031 and CECT 515, resp.) were provided by the Spanish Type Culture Collection (CECT).

To inoculate the growth media, *B. cereus* vegetative cells were grown at 30°C in brain heart infusion broth (BHI; Scharlau Chemie S.A., Barcelona, Spain), until the stationary phase of growth was reached. *L. monocytogenes* vegetative cells were grown at 37°C in tryptic soy broth (TSB; Scharlau Chemie) supplemented (w/v) with 0.6% yeast extract (YE; Scharlau Chemie), until the stationary phase of growth was reached. *E. coli* vegetative cells were grown at 37°C in TSB+YE acidified to pH 5 with ClH (Panreac Química, Barcelona, Spain) until the stationary phase of growth was reached. These growth conditions were chosen as favorable for these microorganisms.

2.2. Optical Density Growth Curves. 100-well microtitre plates were filled with 400 μL of the growth media (BHI for *B. cereus,* TSB+YE for *L. monocytogenes,* and pH 5 TSB+YE for *E. coli*) and were inoculated with the microorganisms and incubated in a Bioscreen C analyzer (Oy Growth Curves Ab Ltd., Helsinki, Finland). Initial concentrations in the growth media were 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 CFU mL^{-1} for *B. cereus,* 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 CFU mL^{-1} for *L. monocytogenes,* and 10^2, 10^4, and 10^6 CFU mL^{-1} for *E. coli*. In order to avoid variability derived from differences in the physiological state of different cultures, all the growth curves from each bacterium were obtained from a single bacterial culture. Growth media were incubated at 30°C for *B. cereus* and at 37°C for *L. monocytogenes* and *E. coli*. At 20 min

Table 1: Primary growth models.

Model	Equation[a]
Three-phase linear	$y = y_0 \quad t \le \lambda$ $y = y_0 + \mu(t-\lambda) \quad \lambda < t < t_s$ $y = y_{\max} \quad t \ge t_s$
Gompertz	$y = y_0 + C\left(e^{\left(-e^{(\mu e(\lambda - t)/C+1)}\right)}\right)$
Logistic	$y = y_0 + \dfrac{C}{1 + e^{(4\mu(\lambda-t)/C+2)}}$
Richards	$y = y_0 + \dfrac{C}{\left(1 + \beta e^{1+\beta} e^{((\mu/C)(1+\beta)(1+1/\beta)(\lambda-t))}\right)^{1/\beta}}$
Baranyi	$y(t) = y_0 + \mu A(t) - \ln\left(1 + \dfrac{e^{\mu A(t)} - 1}{e^C}\right)$ $A(t) = t + \dfrac{1}{\mu}\ln\left(e^{-\mu t} + e^{-\mu\lambda} - e^{-\mu(t+\lambda)}\right)$

[a] y: log count or absorbance at time t; y_0: initial log count or absorbance; μ: maximum growth rate; λ: lag time; t_s: time to reach stationary growth phase; $y_{\max}$: final log count or absorbance; C: increase in log count or absorbance from y_0 to $y_{\max}$; β: model coefficient.

intervals, the optical density (OD) of the samples using a wideband filter (420–580 nm) was measured.

For each combination of microorganism and initial concentration, 25–30 repetitions were performed, except for *B. cereus* 10^1 and 10^2 CFU mL^{-1} initial concentrations, where 13 and 21 repetitions were performed, and for *L. monocytogenes* 10^3, 10^5, and 10^7 CFU mL^{-1} initial concentrations, where 5 repetitions each were performed. Growth curves were obtained by plotting the OD against the exposure time. A total of 345 individual growth curves were generated by absorbance measurements.

2.3. Plate Count Growth Curves. 50 mL flasks of the growth media were inoculated with the microorganisms and incubated with agitation at 500 rpm. Growth media and incubation temperatures were the same as those used for optical density growth curves. Initial concentrations in the growth media were 10^1, 10^3, and 10^5 CFU mL^{-1} for *B. cereus* and 10^2, 10^4, and 10^6 CFU mL^{-1} for *L. monocytogenes* and *E. coli*. At preset time intervals, samples were taken, properly diluted in buffered peptone water (BPW, Scharlau Chemie), and incubated in BHI agar (BHIA, Scharlau Chemie) for 24 h at 30°C for *B. cereus* and in tryptic soy agar (TSA, Scharlau Chemie) + YE for 24 h at 37°C for *L. monocytogenes* and *E. coli*. Growth curves were performed in duplicate.

Growth curves were obtained by plotting log CFU mL^{-1} against the exposure time.

2.4. Mathematical Models. Analyses of the growth curves were performed using five primary growth models. These growth models were based on either linear (derived from the Monod model) or nonlinear (Gompertz, Logistic, Richards, and Baranyi) equations (Table 1) and reparameterized to reflect microbial growth parameters as derived by Zwietering et al. [3].

Curve fitting of three-phase linear, Gompertz, logistic, and Richards models was done using the curve-fitting tool of Matlab 7.0 (Math Works, Natick, USA) with which 95% confidence limits (CL) for growth parameters and r^2, RMSE, and sum of square error (SSE) of fit were calculated.

The curve fitting of Baranyi's equation was done using DMFit 2.0 program and the model of Baranyi and Roberts [23] as kindly provided by Dr. József Baranyi. This program provided standard error for each growth parameter and r^2 and standard error of fit. With these values, 95% CL of growth parameters and RMSE of fit were calculated.

Analysis of variance, medians, and quartiles for box and whisker plots were calculated using StatGraphics (StatPoint Technologies, Warrenton, USA). p values were always lower than 0.05.

3. Results and Discussion

3.1. Optical Density Growth Curves. Figure 1 shows the optical density growth curves plotted with the average OD values at each sampling time of *B. cereus* INRA-AVTZ 415 at 30°C in BHI (a), *L. monocytogenes* CECT 4031 at 37°C in TSB+YE (b), and *E. coli* CECT 515 at 37°C in pH 5 TSB+YE (c) starting at 10^2, 10^4, and 10^6 CFU mL^{-1}. These average growth curves correspond to 25–30 individual growth curves each. The slopes of all the growth curves corresponding to a microorganism were parallel in the exponential growth phase; that is, the growth rates were similar, as they should correspond to different cultures of the same microorganism growing exactly in the same conditions. However, a progressive decrease of lag phase duration was observed as the initial concentration increased. This decrease in lag time can be easily explained because the culture spends less time in reaching a concentration where the absorbance increases. Actually, several authors have recently used simple equations to derive real lag time and provided the initial concentration of microorganisms and the observed lag time from OD measurements [24–26]. The relatively low standard deviation values obtained, especially for growth curves starting at 10^4 and 10^6, are indicative of the repetitivity of the growth curves. It can be noticed that error bars are bigger as the initial concentration decreases, as a consequence of the increased variability among a small population of cells [2].

Table 2 shows the growth parameters given by three-phase linear, Gompertz, logistic, Richards, and Baranyi models for the growth curves of *B. cereus* plotted with the average OD values shown in Figure 1(a). All the models tested provided the values that could be expected for growth parameters of the three growth curves selected of this microorganism. Figure 1(a) shows that all three average growth curves of *B. cereus* were approximately parallel in their exponential growth phases. Although the curve starting at 10^2 CFU mL^{-1} had a slightly lower slope, only logistic and Richards models showed significant differences between this and the other two curves of *B. cereus* starting at higher initial concentrations (Table 2). When comparing the growth rate values given by the different models, three-phase linear

Table 2: Growth parameters and their 95% confidence limits and coefficients of determination (r^2), SSE, and RMSE of fit obtained with different growth models for the average OD growth curves of *Bacillus cereus* INRA-AVTZ 415 at 30°C in BHI inoculating 10^2, 10^4, and 10^6 CFU mL^{-1} shown in Figure 1(a).

Growth model	Initial concentration (CFU mL^{-1})	Lag time (h)	Growth rate (OD units h^{-1})	y_0 (OD units)	C (OD units)	β	r^2	SSE	RMSE
Three-phase linear	10^2	7.81 (7.68–7.94)	0.269 (0.253–0.285)	0.130 (0.042–0.217)	1.056		0.994	0.0034	0.0207
	10^4	5.47 (5.29–5.66)	0.304 (0.274–0.334)	0.140 (0.019–0.261)	0.999		0.989	0.0042	0.0268
	10^6	2.82 (2.68–2.95)	0.293 (0.273–0.312)	0.180 (0.027–0.332)	0.975		0.994	0.0032	0.0215
Gompertz	10^2	7.95 (7.87–8.03)	0.312 (0.301–0.329)	0.117 (0.109–0.124)	1.107 (1.095–1.120)		0.999	0.0166	0.0179
	10^4	5.50 (5.35–5.64)	0.342 (0.314–0.370)	0.118 (0.103–0.133)	1.078 (1.050–1.106)		0.996	0.0281	0.0283
	10^6	2.87 (2.69–3.04)	0.349 (0.315–0.382)	0.142 (0.119–0.166)	1.057 (1.019–1.095)		0.995	0.0252	0.0311
Logistic	10^2	8.01 (7.97–8.06)	0.313 (0.307–0.319)	0.105 (0.102–0.109)	1.104 (1.099–1.110)		0.999	0.0031	0.0077
	10^4	5.56 (5.47–5.65)	0.345 (0.329–0.360)	0.105 (0.096–0.150)	1.070 (1.056–1.085)		0.999	0.0081	0.0152
	10^6	2.87 (2.75–2.99)	0.347 (0.329–0.365)	0.114 (0.098–0.110)	1.066 (1.045–1.088)		0.999	0.0070	0.0164
Richards	10^2	8.00 (7.96–8.05)	0.303 (0.285–0.321)	0.106 (0.103–0.110)	1.104 (1.099–1.110)	0.911 (0.769–1.054)	0.999	0.0030	0.0076
	10^4	5.63 (5.54–5.72)	0.401 (0.373–0.429)	0.096 (0.086–0.100)	1.071 (1.058–1.084)	1.693 (1.257–2.130)	0.999	0.0060	0.0132
	10^6	2.85 (2.75–2.95)	0.420 (0.400–0.439)	0.071 (0.048–0.090)	1.099 (1.076–1.122)	2.490 (1.823–3.158)	0.999	0.0029	0.0108
Baranyi	10^2	7.80 (7.70–7.90)	0.275 (0.264–0.286)	0.155	1.047 (1.040–1.054)		0.999		0.0155
	10^4	5.40 (5.29–5.51)	0.307 (0.291–0.323)	0.141	1.020 (1.010–1.030)		0.999		0.0178
	10^6	2.76 (2.66–2.87)	0.312 (0.298–0.326)	0.152	1.015 (1.006–1.023)		0.999		0.0148

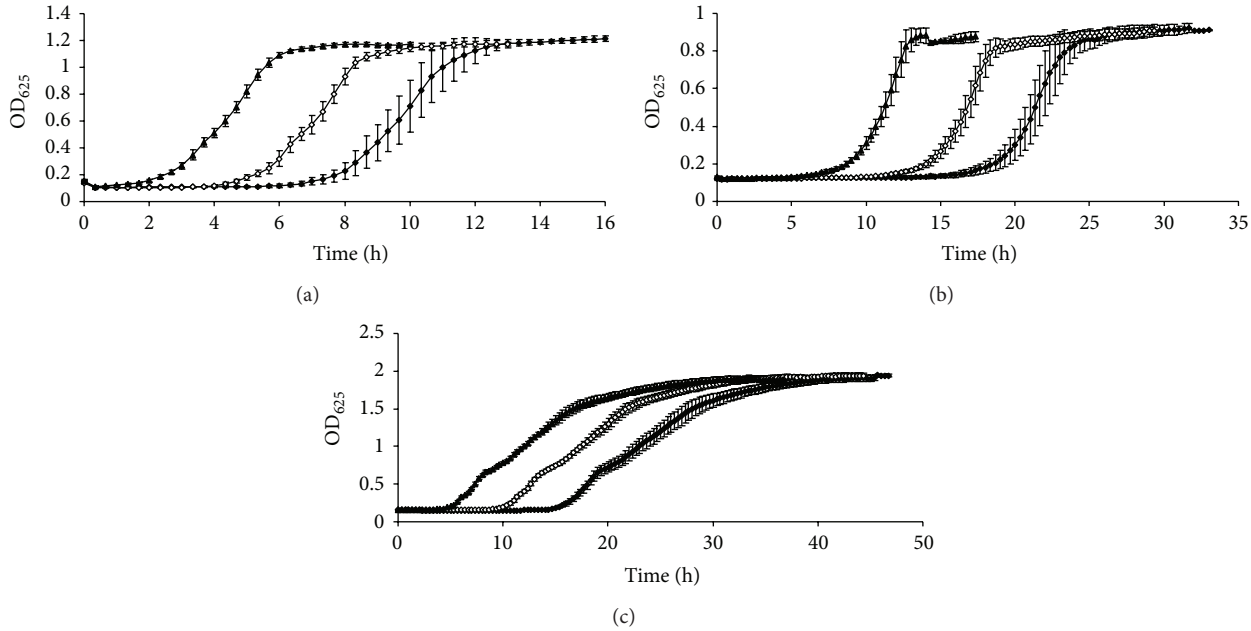

Figure 1: Optical density growth curves plotted with the average OD values (±standard deviation) at each sampling time of *Bacillus cereus* INRA-AVTZ 415 at 30°C in BHI (a), *Listeria monocytogenes* CECT 4031 at 37°C in TSB+YE (b), and *Escherichia coli* CECT 515 at 37°C in pH 5 TSB+YE (c). Initial number of microorganisms: (♦) 10^2 CFU mL^{-1}; (◊) 10^4 CFU mL^{-1}; (▲) 10^6 CFU mL^{-1}.

Table 3: Growth parameters and their 95% confidence limits and coefficients of determination (r^2), SSE, and RMSE of fit obtained with different growth models for the average OD growth curves of *Listeria monocytogenes* CECT 4031 at 37°C in TSB+YE inoculating 10^2, 10^4, and 10^6 CFU mL^{-1} shown in Figure 1(b).

Growth model	Initial concentration (CFU mL^{-1})	Lag time (h)	Growth rate (OD units h^{-1})	y_0 (OD units)	C (OD units)	β	r^2	SSE	RMSE
Three-phase linear	10^2	19.11 (18.96–19.27)	0.163 (0.154–0.173)	0.142 (0.069–0.214)	0.742		0.994	0.0013	0.0127
	10^4	14.61 (14.42–14.81)	0.173 (0.159–0.187)	0.143 (0.065–0.222)	0.724		0.989	0.0026	0.0181
	10^6	8.79 (8.23–9.35)	0.150 (0.126–0.174)	0.149 (0.072–0.227)	0.712		0.930	0.0490	0.0614
Gompertz	10^2	18.99 (18.87–19.10)	0.169 (0.161–0.177)	0.132 (0.128–0.136)	0.784 (0.776–0.792)		0.998	0.0221	0.0153
	10^4	14.46 (14.31–14.62)	0.179 (0.167–0.191)	0.133 (0.127–0.139)	0.757 (0.746–0.768)		0.997	0.0353	0.0204
	10^6	9.33 (9.09–9.56)	0.218 (0.190–0.245)	0.141 (0.129–0.154)	0.755 (0.726–0.783)		0.990	0.0491	0.0320
Logistic	10^2	19.06 (18.99–19.12)	0.168 (0.163–0.171)	0.128 (0.126–0.130)	0.779 (0.775–0.783)		0.999	0.0052	0.0074
	10^4	14.55 (14.43–14.67)	0.178 (0.170–0.186)	0.128 (0.123–0.132)	0.755 (0.748–0.762)		0.999	0.0160	0.0137
	10^6	9.40 (9.22–9.58)	0.217 (0.199–0.235)	0.135 (0.126–0.144)	0.747 (0.730–0.765)		0.996	0.0227	0.0218
Richards	10^2	19.12 (19.05–19.19)	0.183 (0.175–0.191)	0.126 (0.124–0.128)	0.778 (0.774–0.781)	1.333 (1.152–1.514)	0.999	0.0045	0.0069
	10^4	14.74 (14.61–14.87)	0.219 (0.205–0.233)	0.123 (0.119–0.128)	0.754 (0.748–0.760)	2.040 (1.565–2.515)	0.999	0.0123	0.0121
	10^6	9.93 (9.81–10.06)	0.318 (0.303–0.333)	0.119 (0.115–0.123)	0.748 (0.742–0.754)	6.730 (4.925–8.536)	0.999	0.0028	0.0078
Baranyi	10^2	18.79 (18.65–18.93)	0.148 (0.142–0.155)	0.120	0.781 (0.776–0.786)		0.998		0.0138
	10^4	14.04 (13.87–14.21)	0.163 (0.153–0.173)	0.130	0.745 (0.739–0.752)		0.997		0.0178
	10^6	8.95 (8.75–9.15)	0.201 (0.183–0.218)	0.139	0.731 (0.718–0.743)		0.995		0.0219

model gave the lowest values (0.27–0.30 OD units h^{-1}), followed by Baranyi model (0.28–0.31 OD units h^{-1}), logistic and Gompertz (0.31–0.35 OD units h^{-1}), and Richards model (0.30–0.42 OD units h^{-1}). Also, all models provided shorter lag phases at higher initial concentrations, as shown in Figure 1(a). Initial absorbance (y_0) had values between 0.071 and 0.180 for all growth curves and models. The increase in absorbance from initial to final optical density (C) had values between 0.975 and 1.107 for all growth curves and models. Hence all models seemed to perform adequately, providing expected growth parameter values for these growth curves. r^2 values were higher than 0.995 for all growth curves and all models, except for three-phase linear model, which gave values as low as 0.989, hence being the model which provided worst fit to the data, as also it could be expected from a model consisting of three straight lines. All models gave RMSE values lower than 0.035.

Table 3 shows the growth parameters given by the five models for the growth curves of *L. monocytogenes* shown in Figure 1(b). Similar results were obtained, although in this case the only model which did not provide significant differences for the three growth rate values for the three initial concentrations tested was the three-phase linear. Again, the same order was obtained, three-phase linear model giving the lowest values (0.15–0.17 OD units h^{-1}), followed by Baranyi model (0.15–0.20 OD units h^{-1}), logistic and Gompertz (0.17–0.22 OD units h^{-1}), and Richards model giving the highest values (0.18–0.32 OD units h^{-1}). Also, all models provided shorter lag phases at higher initial concentrations. y_0 values were between 0.119 and 0.149 and C values between 0.712 and 0.784. Again, r^2 values were higher than 0.990 for all growth curves and all models, except for three-phase linear model, which gave values as low as 0.930. Three-phase linear model also gave RMSE values as high as 0.061.

Table 4 shows the growth parameters given by the five models under study for the growth curves of *E. coli* shown in Figure 1(c). In this case, some unexpected results were obtained. Three-phase linear, Gompertz, logistic, and Baranyi

Table 4: Growth parameters obtained and their 95% confidence limits and coefficients of determination (r^2), SSE, and RMSE of fit with different growth models for the average OD growth curves of *Escherichia coli* CECT 515 at 37°C in pH 5 TSB+YE inoculating 10^2, 10^4, and 10^6 CFU mL^{-1} shown in Figure 1(c).

Growth model	Initial concentration (CFU mL^{-1})	Lag time (h)	Growth rate (OD units h^{-1})	y_0 (OD units)	C (OD units)	β	r^2	SSE	RMSE
Three-phase linear	10^2	14.80 (14.60–15.00)	0.103 (0.100–0.105)	0.161 (0.115–0.207)	1.622		0.995	0.0200	0.0250
	10^4	9.70 (9.53–9.86)	0.110 (0.107–0.112)	0.167 (0.111–0.223)	1.656		0.996	0.0150	0.0220
	10^6	4.44 (4.27–4.61)	0.111 (0.108–0.113)	0.176 (0.113–0.239)	1.600		0.996	0.0150	0.0224
Gompertz	10^2	15.46 (15.29–15.62)	0.117 (0.115–0.119)	0.144 (0.137–0.152)	1.794 (1.780–1.807)		0.999	0.0700	0.0227
	10^4	10.29 (10.13–10.46)	0.125 (0.123–0.128)	0.146 (0.137–0.155)	1.799 (1.785–1.812)		0.999	0.0663	0.0227
	10^6	4.65 (4.42–4.89)	0.125 (0.122–0.127)	0.123 (0.106–0.140)	1.783 (1.761–1.805)		0.999	0.0597	0.0237
Logistic	10^2	15.51 (15.12–15.90)	0.117 (0.113–0.121)	0.105 (0.088–0.121)	1.784 (1.758–1.810)		0.997	0.2544	0.0433
	10^4	9.95 (9.56–10.33)	0.123 (0.119–0.126)	0.078 (0.057–0.094)	1.834 (1.808–1.860)		0.997	0.1825	0.0376
	10^6	3.67 (3.46–3.89)	0.120 (0.117–0.123)	0.000[a]	1.875 (1.864–1.887)		0.997	0.1232	0.0339
Richards	10^2	15.46 (15.18–15.73)	0.00008 (−0.016–0.016)	0.144 (0.137–0.152)	1.794 (1.772–1.816)	0.00024 (−0.049–0.049)	0.999	0.0670	0.0228
	10^4	10.29 (9.98–10.61)	0.0001 (−0.063–0.063)	0.146 (0.135–0.156)	1.799 (1.778–1.819)	0.00041 (−0.185–0.186)	0.999	0.0663	0.0228
	10^6	4.65 (4.41–4.90)	0.0002 (−0.107–0.107)	0.123 (0.086–0.159)	1.783 (1.743–1.824)	0.00062 (−0.316–0.317)	0.999	0.0598	0.0239
Baranyi	10^2	14.31 (13.87–14.75)	0.101 (0.097–0.105)	0.126	1.742 (1.727–1.756)		0.996		0.0445
	10^4	8.96 (8.50–9.41)	0.107 (0.103–0.111)	0.115	1.777 (1.763–1.789)		0.996		0.0445
	10^6	2.72 (2.07–3.38)	0.104 (0.099–0.109)	0.060	1.791 (1.776–1.806)		0.994		0.0483

[a]Value fixed at bound.

models behaved as previously described for *B. cereus* and *L. monocytogenes*. Each model provided very similar values for the growth rates of the three growth curves, with only slight differences among them. However, only logistic and Baranyi models did not provide significant differences. For this microorganism, again, the three-phase linear model gave the lowest value for the growth rate (0.10–0.11 OD units h^{-1}), but in this case together with Baranyi model, and again logistic and Gompertz models gave higher values (0.12–0.13 OD units h^{-1}). However, Richards model gave unexpectedly low values for growth rates (<0.001 OD units h^{-1}). All models provided shorter lag phases at higher initial concentrations. y_0 values were between 0.060 and 0.176 for all growth curves and models, except for the logistic model, which gave an exceptional low y_0 value of 0.000 (fixed at bound) for growth curve starting at 10^6 CFU mL^{-1}. Logistic model gave values of SSE higher than 0.1 for all three growth curves of *E. coli*, which were almost twice the values obtained for any other growth curve shown in Table 2, 3, or 4. In this case all r^2 values were higher than 0.99 for all growth curves and all models, including the three-phase linear model.

After the results shown in Figure 1 and Tables 2, 3, and 4, the growth models selected for the rest of the investigation were three-phase linear, Gompertz, and Baranyi. The three selected models provided expected values for the growth parameters and fitted data properly, as shown by the statistics analysed, even in the case of the three-phase linear model, which was the one to provide worse goodness of fit, that is, lowest r^2 and highest RMSE values. Richards and logistic models were disregarded because they were not able to fit properly all these typical growth curves; that is, they gave abnormal values for growth parameters in some occasions.

Each model had a trend in providing higher or lower values, three-phase linear model giving consistently the lowest

Table 5: Growth rate values and their 95% confidence limits and coefficients of determination (r^2) and RMSE of fit obtained with three-phase linear, Gompertz, and Baranyi growth models for the growth curves of *Escherichia coli* CECT 515 at 37°C in pH 5 TSB+YE inoculating 10^2, 10^4, and 10^6 CFU mL^{-1}.

Initial concentration (CFU mL^{-1})	Three-phase linear model			Gompertz model			Baranyi model		
	Growth rate (OD units h^{-1})	r^2	RMSE	Growth rate (OD units h^{-1})	r^2	RMSE	Growth rate (OD units h^{-1})	r^2	RMSE
10^2	0.076 (0.073–0.078)	0.988	0.0425	0.094 (0.092–0.097)	0.997	0.0346	0.091	0.993	0.0516
	0.086 (0.084–0.088)	0.994	0.0295	0.100 (0.097–0.102)	0.998	0.0296	0.112	0.995	0.0469
	0.091 (0.089–0.093)	0.995	0.0284	0.106 (0.103–0.108)	0.998	0.0283	0.112	0.995	0.0478
	0.100 (0.096–0.105)	0.982	0.0516	0.118 (0.115–0.121)	0.998	0.0317	0.112	0.995	0.0456
	0.082 (0.078–0.085)	0.990	0.0414	0.100 (0.097–0.102)	0.998	0.0319	0.116	0.996	0.0412
	0.091 (0.088–0.094)	0.988	0.0478	0.114 (0.112–0.116)	0.999	0.0203	0.115	0.995	0.0453
	0.095 (0.093–0.097)	0.994	0.0348	0.113 (0.112–0.115)	0.999	0.0179	0.113	0.995	0.0482
	0.097 (0.095–0.099)	0.996	0.0230	0.111 (0.109–0.113)	0.999	0.0239	0.113	0.995	0.0465
	0.102 (0.100–0.104)	0.998	0.0173	0.116 (0.114–0.118)	0.999	0.0204	0.111	0.995	0.0489
	0.087 (0.085–0.089)	0.994	0.0300	0.100 (0.098–0.103)	0.998	0.0297	0.114	0.995	0.0475
	0.109 (0.107–0.111)	0.997	0.0215	0.125 (0.122–0.127)	0.999	0.0217	0.109	0.995	0.0458
	0.111 (0.108–0.114)	0.995	0.0285	0.126 (0.124–0.129)	0.999	0.0234	0.111	0.995	0.0444
	0.114 (0.111–0.117)	0.994	0.0319	0.132 (0.129–0.136)	0.999	0.0269	0.113	0.994	0.0499
	0.110 (0.106–0.114)	0.986	0.0516	0.132 (0.129–0.136)	0.999	0.0286	0.109	0.993	0.0543
	0.116 (0.113–0.119)	0.994	0.0330	0.136 (0.133–0.139)	0.999	0.0224	0.104	0.994	0.0516
	0.104 (0.101–0.106)	0.995	0.0318	0.120 (0.117–0.123)	0.998	0.0292	0.003	0.156	0.9944
	0.107 (0.105–0.109)	0.997	0.0223	0.122 (0.120–0.124)	0.999	0.0215	0.110	0.995	0.0465
	0.106 (0.104–0.109)	0.994	0.0332	0.122 (0.120–0.125)	0.999	0.0297	0.115	0.996	0.0443
	0.106 (0.103–0.108)	0.993	0.0378	0.123 (0.120–0.126)	0.999	0.0264	0.109	0.995	0.0456
	0.112 (0.106–0.118)	0.977	0.0629	0.136 (0.132–0.139)	0.999	0.0282	0.091	0.993	0.0501
	0.085 (0.083–0.087)	0.990	0.0415	0.106 (0.104–0.108)	0.999	0.0240	0.083	0.991	0.0576
	0.108 (0.106–0.110)	0.997	0.0223	0.121 (0.119–0.123)	0.999	0.0217	0.103	0.994	0.0499
	0.084 (0.081–0.088)	0.984	0.0507	0.098 (0.095–0.102)	0.996	0.0440	0.098	0.994	0.0500
	0.108 (0.106–0.110)	0.996	0.0260	0.125 (0.122–0.128)	0.999	0.0261	0.109	0.995	0.0458
	0.108 (0.106–0.111)	0.996	0.0277	0.125 (0.123–0.128)	0.999	0.0262	0.098	0.993	0.0525
	0.110 (0.106–0.114)	0.988	0.0484	0.132 (0.129–0.135)	0.999	0.0257	0.101	0.994	0.0498
	0.109 (0.106–0.111)	0.994	0.0338	0.128 (0.125–0.131)	0.999	0.0259	0.102	0.993	0.0517
	0.102 (0.100–0.104)	0.995	0.0264	0.116 (0.114–0.119)	0.999	0.0252	0.102	0.993	0.0536
	0.104 (0.101–0.108)	0.991	0.0394	0.121 (0.118–0.124)	0.998	0.0329	0.104	0.994	0.0499
	0.118 (0.115–0.121)	0.996	0.0275	0.137 (0.135–0.140)	0.999	0.0216	0.095	0.993	0.0510
10^4	0.090 (0.088–0.093)	0.993	0.0331	0.105 (0.102–0.107)	0.998	0.0319	0.090	0.996	0.0432
	0.099 (0.096–0.102)	0.991	0.0429	0.120 (0.117–0.1229)	0.998	0.0292	0.106	0.996	0.0437
	0.102 (0.099–0.104)	0.995	0.0283	0.117 (0.115–0.119)	0.999	0.0283	0.101	0.996	0.0477
	0.101 (0.099–0.103)	0.995	0.0304	0.117 (0.115–0.120)	0.998	0.0290	0.104	0.996	0.0418
	0.101 (0.099–0.104)	0.995	0.0283	0.115 (0.112–0.118)	0.998	0.0304	0.102	0.996	0.0452
	0.102 (0.100–0.104)	0.996	0.0295	0.118 (0.115–0.121)	0.998	0.0302	0.105	0.996	0.0433
	0.095 (0.093–0.098)	0.989	0.0464	0.116 (0.113–0.119)	0.998	0.0308	0.101	0.996	0.0476
	0.101 (0.098–0.103)	0.995	0.0279	0.115 (0.112–0.117)	0.998	0.0283	0.100	0.996	0.0450
	0.102 (0.100–0.104)	0.996	0.0257	0.116 (0.114–0.118)	0.999	0.0216	0.100	0.995	0.0477
	0.074 (0.070–0.079)	0.950	0.1039	0.116 (0.113–0.118)	0.999	0.0249	0.100	0.995	0.0479
	0.094 (0.091–0.096)	0.993	0.0315	0.110 (0.108–0.113)	0.998	0.0264	0.096	0.995	0.0465
	0.098 (0.095–0.100)	0.992	0.0431	0.118 (0.115–0.120)	0.999	0.0258	0.103	0.996	0.0431
	0.113 (0.111–0.116)	0.996	0.0264	0.131 (0.128–0.134)	0.999	0.0267	0.116	0.997	0.0427
	0.111 (0.109–0.114)	0.995	0.0278	0.126 (0.123–0.130)	0.998	0.0286	0.111	0.996	0.0453

Table 5: Continued.

Initial concentration (CFU mL^{-1})	Three-phase linear model			Gompertz model			Baranyi model		
	Growth rate (OD units h^{-1})	r^2	RMSE	Growth rate (OD units h^{-1})	r^2	RMSE	Growth rate (OD units h^{-1})	r^2	RMSE
	0.108 (0.106–0.110)	0.997	0.0231	0.123 (0.121–0.126)	0.999	0.0244	0.108	0.996	0.0444
	0.112 (0.109–0.115)	0.992	0.0362	0.128 (0.124–0.131)	0.998	0.0338	0.113	0.996	0.0452
	0.110 (0.107–0.112)	0.996	0.0256	0.125 (0.122–0.128)	0.999	0.0280	0.111	0.996	0.0429
	0.105 (0.102–0.107)	0.995	0.0302	0.123 (0.121–0.126)	0.999	0.0239	0.107	0.996	0.0460
	0.111 (0.107–0.116)	0.982	0.0638	0.140 (0.137–0.143)	0.999	0.0275	0.124	0.996	0.0443
	0.119 (0.117–0.121)	0.997	0.0225	0.137 (0.134–0.140)	0.999	0.0265	0.122	0.997	0.0400
	0.117 (0.113–0.120)	0.992	0.0399	0.140 (0.137–0.143)	0.999	0.0236	0.123	0.996	0.0454
	0.120 (0.118–0.122)	0.998	0.0218	0.138 (0.135–0.142)	0.999	0.0260	0.123	0.997	0.0401
	0.120 (0.117–0.122)	0.996	0.0274	0.137 (0.134–0.140)	0.999	0.0271	0.120	0.996	0.0471
	0.116 (0.113–0.119)	0.995	0.0304	0.132 (0.129–0.135)	0.999	0.0249	0.117	0.997	0.0397
	0.120 (0.117–0.123)	0.994	0.0329	0.137 (0.133–0.141)	0.998	0.0326	0.123	0.997	0.0424
	0.117 (0.114–0.119)	0.997	0.0247	0.135 (0.132–0.138)	0.999	0.0239	0.121	0.997	0.0374
	0.105 (0.101–0.109)	0.980	0.0715	0.137 (0.133–0.141)	0.998	0.0292	0.123	0.997	0.0371
	0.112 (0.110–0.114)	0.997	0.0216	0.128 (0.125–0.130)	0.999	0.0218	0.112	0.996	0.0458
	0.120 (0.117–0.122)	0.996	0.0278	0.137 (0.134–0.140)	0.999	0.0249	0.120	0.996	0.0449
	0.109 (0.106–0.112)	0.993	0.0318	0.124 (0.121–0.126)	0.999	0.0246	0.105	0.994	0.0531
10^6	0.092 (0.089–0.096)	0.989	0.0400	0.112 (0.109–0.114)	0.998	0.0269	0.091	0.993	0.0516
	0.114 (0.111–0.117)	0.994	0.0302	0.130 (0.126–0.134)	0.998	0.0325	0.112	0.995	0.0469
	0.101 (0.096–0.105)	0.979	0.0667	0.130 (0.127–0.134)	0.998	0.0278	0.112	0.995	0.0478
	0.115 (0.112–0.117)	0.996	0.0248	0.129 (0.126–0.132)	0.998	0.0267	0.112	0.995	0.0456
	0.112 (0.109–0.115)	0.995	0.0310	0.131 (0.128–0.135)	0.998	0.0303	0.116	0.996	0.0412
	0.115 (0.113–0.117)	0.996	0.0245	0.132 (0.129–0.135)	0.999	0.0259	0.115	0.995	0.0453
	0.117 (0.114–0.119)	0.997	0.0243	0.131 (0.128–0.135)	0.998	0.0280	0.113	0.995	0.0482
	0.113 (0.110–0.115)	0.995	0.0319	0.130 (0.126–0.134)	0.998	0.0321	0.113	0.995	0.0465
	0.114 (0.112–0.117)	0.995	0.0294	0.130 (0.126–0.133)	0.998	0.0298	0.111	0.995	0.0489
	0.114 (0.111–0.117)	0.995	0.0282	0.132 (0.129–0.134)	0.998	0.0284	0.114	0.995	0.0475
	0.111 (0.108–0.113)	0.997	0.0223	0.127 (0.124–0.130)	0.998	0.0269	0.109	0.995	0.0458
	0.112 (0.109–0.115)	0.995	0.0292	0.128 (0.125–0.132)	0.998	0.0309	0.111	0.995	0.0444
	0.116 (0.114–0.118)	0.997	0.0227	0.131 (0.128–0.135)	0.998	0.0261	0.113	0.994	0.0499
	0.069 (0.063–0.074)	0.903	0.1575	0.129 (0.127–0.132)	0.999	0.0248	0.109	0.993	0.0543
	0.108 (0.106–0.111)	0.996	0.0250	0.123 (0.120–0.126)	0.999	0.0243	0.104	0.994	0.0516
	0.120 (0.117–0.122)	0.995	0.0289	0.137 (0.133–0.140)	0.998	0.0291	0.003	0.156	0.9944
	0.112 (0.110–0.115)	0.996	0.0250	0.127 (0.124–0.131)	0.998	0.0289	0.110	0.995	0.0465
	0.113 (0.111–0.116)	0.996	0.0279	0.132 (0.129–0.136)	0.998	0.0284	0.115	0.996	0.0443
	0.102 (0.098–0.106)	0.987	0.0494	0.126 (0.123–0.129)	0.998	0.0261	0.109	0.995	0.0456
	0.086 (0.082–0.089)	0.982	0.0543	0.109 (0.106–0.112)	0.998	0.0278	0.091	0.993	0.0501
	0.079 (0.076–0.083)	0.975	0.0603	0.101 (0.098–0.105)	0.997	0.0353	0.083	0.991	0.0576
	0.106 (0.104–0.109)	0.995	0.0286	0.122 (0.119–0.125)	0.998	0.0281	0.103	0.994	0.0499
	0.102 (0.099–0.105)	0.993	0.0306	0.117 (0.114–0.119)	0.998	0.0261	0.098	0.994	0.0500
	0.111 (0.108–0.113)	0.996	0.0234	0.127 (0.124–0.130)	0.999	0.0251	0.109	0.995	0.0458
	0.102 (0.099–0.105)	0.992	0.0345	0.119 (0.116–0.121)	0.999	0.0240	0.098	0.993	0.0525
	0.104 (0.101–0.107)	0.993	0.0293	0.119 (0.116–0.122)	0.998	0.0258	0.101	0.994	0.0498
	0.071 (0.066–0.076)	0.931	0.1209	0.121 (0.118–0.124)	0.998	0.0250	0.102	0.993	0.0517
	0.105 (0.102–0.108)	0.994	0.0312	0.122 (0.119–0.125)	0.998	0.0266	0.102	0.993	0.0536
	0.106 (0.103–0.109)	0.993	0.0341	0.123 (0.120–0.126)	0.998	0.0270	0.104	0.994	0.0499
	0.094 (0.091–0.098)	0.989	0.0399	0.114 (0.111–0.117)	0.998	0.0292	0.095	0.993	0.0510

values, followed by Baranyi and Gompertz models, in this order, for both the growth rate and the lag phase. In a previous comparison of these three models, Buchanan et al. [2] already noticed and explained this effect on the basis of the nature of each model. These authors also highlighted the correlation existing between lag phase duration and specific growth rate values. This gives an explanation for the differences found in the values provided for growth rate and lag phase among these predictive models.

Hence, it seems that, depending on the predictive model chosen, values for growth rates and lag times will be consistently higher or lower. Then the question is which growth model can be considered the best for describing the true population growth and why. This can be answered with a deep analysis of the analytical aspects of the models, mechanistic elements, number of parameters, fitting properties, and so forth. However, such analyses have already been performed in the past [3], but researchers continue to use and compare different growth models [5, 12], probably because concluding results have not been reached yet.

In this context, our viewpoint is that the best performing model is the one which, for the growth parameters of different cultures of one microbial strain growing under exactly the same conditions and with the same precultural history, gives closer values.

With this purpose, an extensive analysis of growth curves was performed with the three selected growth models. Only growth rate was considered at this stage, since this parameter should be similar for all growth curves of the same microbial strain, even when starting from different initial inocula levels. A total of 345 individual growth curves were analysed, including those individual growth curves used to build the average OD growth curves shown in Figure 1.

Table 5 shows, as an example, growth rate values obtained for all *E. coli* individual growth curves with three-phase linear, Gompertz, and Baranyi growth models. With these data and those for the individual growth curves of *B. cereus* and *L. monocytogenes,* extensive statistical analyses were performed, including analyses of variance, medians, and quartiles. Analyses of variance showed that initial concentration did not influence growth rate and that significant differences were found among growth rate values given by the different models, as already pointed out. The statistical analyses also showed that, within models, growth rate data were not normally distributed, and significant differences were also found among median growth rate values.

The last step of this research was to analyse variation of growth rate values. Table 6 shows average and standard deviation for growth rate of all three microorganisms as obtained by three-phase linear, Gompertz, and Baranyi models, and Figure 2 shows box and whiskers plots for the growth rate of the three microorganisms. These plots are based on the median and withstand perturbations caused by outliers better than plots based on the average. Since growth rate data were not normally distributed, box and whiskers plots are more appropriate than average and standard deviation shown in Table 6.

Table 6: Average ± standard deviation of growth rate values (OD units h^{-1}) obtained with three-phase linear, Gompertz, and Baranyi growth models for all the growth curves of *Bacillus cereus* INRA-AVTZ 415 at 30°C in BHI, *Listeria monocytogenes* CECT 4031 at 37°C in TSB+YE, and *Escherichia coli* CECT 515 at 37°C in pH 5 TSB+YE.

Growth model	*B. cereus*	*L. monocytogenes*	*E. coli*
Three-phase linear	0.226 ± 0.062	0.170 ± 0.022	0.104 ± 0.011
Gompertz	0.364 ± 0.627	0.296 ± 0.941	0.123 ± 0.011
Baranyi	0.261 ± 0.085	0.164 ± 0.042	0.105 ± 0.018

The results shown in Figure 2 clearly show that Gompertz model has a higher degree of variation in the growth rate values than the three-phase linear and Baranyi models. Gompertz model also generated some outlier growth rate values which extended the "whiskers" several units in the case of *B. cereus* and *L. monocytogenes*. It is certainly possible to obtain correct values for these outliers by changing the initial values when performing the nonlinear regression, but the purpose of this research was not to optimize the models, but to test them in order to choose the model that performs best. Hence initial values were not changed. Three-phase linear model provided slightly less variation than Baranyi, although the goodness of fit of this model is considerably worse than that of Baranyi model.

3.2. Plate Count Growth Curves. In order to double check the results obtained, several plate count growth curves of these same microorganisms were modelled with the five growth models. Figure 3 shows the growth curves plotted with the average log CFU mL^{-1} at each sampling time of *B. cereus* INRA-AVTZ 415 at 30°C in BHI (a), *L. monocytogenes* CECT 4031 at 37°C in TSB+YE (b), and *E. coli* CECT 515 at 37°C in pH 5 TSB+YE (c) starting at different initial concentration of microorganisms. As usual for plate count growth curves, these curves included less data per curve and did not show data points in all growth phases. In this way, models were forced into the common situation of scarce data points. Tables 7, 8, and 9 show the growth parameters given by three-phase linear, Gompertz, logistic, Richards, and Baranyi models for the growth curves of *B. cereus, L. monocytogenes,* and *E. coli* shown in Figures 3(a), 3(b), and 3(c), respectively.

Similar results to those obtained with absorbance data were obtained when modelling the data from plate count growth curves. Three-phase linear model gave the lowest values for growth rates and lag times, followed by Baranyi, Gompertz, and logistic, in that order, for all three microorganisms. For these plate count growth curves, Richards model was not able to provide satisfactory values for growth rate for any of the curves. It is worth noting that some of the growth curves did not reach the stationary growth phase, and both three-phase linear and Baranyi models did not provide values for maximum population levels in these cases, while all other models provided uncertain estimations for this parameter.

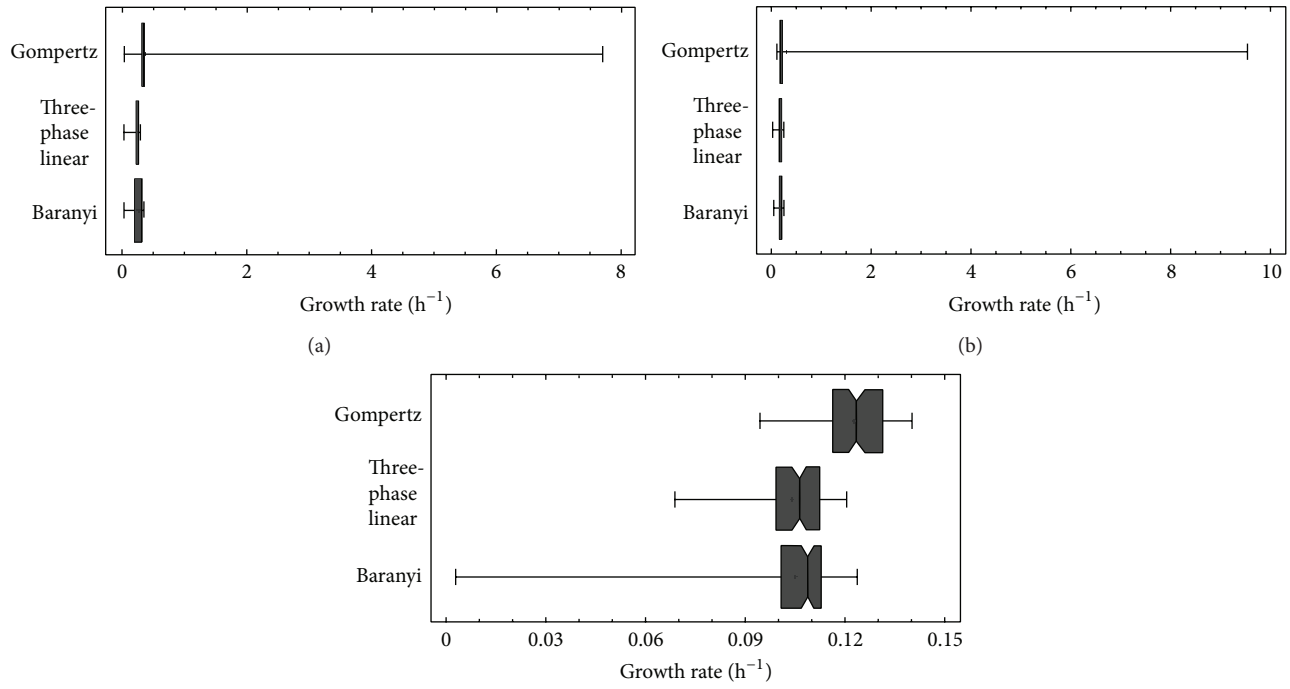

Figure 2: Box and whiskers plots for growth rate values of *Bacillus cereus* INRA-AVTZ 415 at 30°C in BHI (a), *Listeria monocytogenes* CECT 4031 at 37°C in TSB+YE (b), and *Escherichia coli* CECT 515 at 37°C in pH 5 TSB+YE (c).

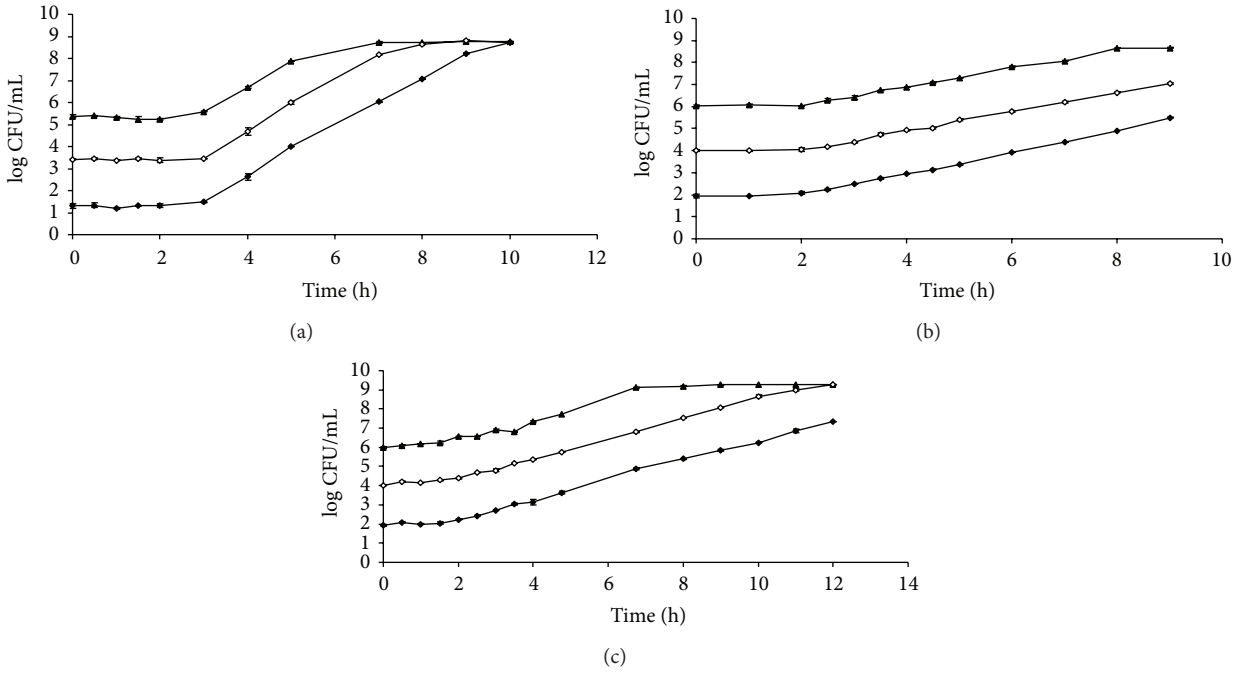

Figure 3: Plate count growth curves plotted with the average plate count values (±standard deviation) at each sampling time of *Bacillus cereus* INRA-AVTZ 415 at 30°C in BHI (a), *Listeria monocytogenes* CECT 4031 at 37°C in TSB+YE (b), and *Escherichia coli* CECT 515 at 37°C in pH 5 TSB+YE (c). Initial number of microorganisms: (♦) 10^2 CFU mL^{-1}; (◊) 10^4 CFU mL^{-1}; (▲) 10^6 CFU mL^{-1}.

Table 7: Growth parameters and their 95% confidence limits and coefficients of determination (r^2), SSE, and RMSE of fit obtained with different growth models for average plate count growth curves of *Bacillus cereus* INRA-AVTZ 415 at 30°C in BHI inoculating 10, 10^3, and 10^5 CFU mL^{-1} shown in Figure 3(a).

Growth model	Initial concentration (CFU mL^{-1})	Lag time (h)	Growth rate (log cycles h^{-1})	y_0 (log CFU mL^{-1})	C (log cycles)	β	r^2	SSE	RMSE
Three-phase linear	10	2.75 (2.59–2.91)	1.112 (1.073–1.115)	1.343 (1.202–1.484)	7.701		0.993	0.2880	0.2400
	10^3	2.92 (2.79–3.05)	1.182 (1.121–1.138)	3.414 (3.338–3.490)	5.337		0.927	0.7295	0.4931
	10^5	2.77 (2.64–2.91)	1.143 (1.049–1.232)	5.367 (5.289–5.444)	3.372		0.920	0.8099	0.4025
Gompertz	10	3.06 (2.59–3.52)	1.276 (1.112–1.440)	1.211 (0.983–1.440)	9.049 (7.519–10.580)		0.996	0.2846	0.1886
	10^3	3.30 (3.08–3.53)	1.598 (1.380–1.816)	3.403 (3.302–3.504)	5.521 (5.284–5.757)		0.998	0.0811	0.1007
	10^5	3.00 (2.82–3.17)	1.386 (1.206–1.565)	5.319 (5.257–5.382)	3.469 (3.363–3.574)		0.999	0.0301	0.0613
Logistic	10	3.04 (2.12–3.96)	1.318 (1.081–1.556)	0.914 (0.408–1.421)	8.529 (6.904–10.150)		0.992	0.5385	0.2595
	10^3	3.39 (3.00–3.79)	1.651 (1.296–2.006)	3.330 (3.154–3.507)	5.433 (5.112–5.754)		0.996	0.1747	0.1478
	10^5	3.10 (2.84–3.36)	1.451 (1.193–1.709)	5.288 (5.194–5.381)	3.461 (3.320–3.603)		0.997	0.0551	0.0830
Richards	10	3.06 (1.92–4.19)	0.0006 (−4.543–4.544)	1.211 (0.779–1.643)	9.049 (5.176–12.920)	0.00017 (−1.310–1.311)	0.995	0.2846	0.2016
	10^3	3.30 (3.05–3.55)	0.004 (−2.950–2.958)	3.403 (3.288–3.519)	5.520 (5.229–5.812)	0.0009 (−0.681–0.683)	0.998	0.0811	0.1070
	10^5	3.02 (2.82–3.21)	0.536 (−1.052–2.125)	5.316 (5.247–5.385)	3.460 (3.350–3.571)	0.181 (−0.508–0.871)	0.998	0.0275	0.0627
Baranyi	10	2.88 (2.39–3.37)	1.187 (1.054–1.324)	1.172	7.869		0.996		0.1792
	10^3	3.09 (2.77–3.41)	1.313 (1.137–1.324)	3.360	5.387		0.997		0.1248
	10^5	2.95 (2.75–3.15)	1.275 (1.121–1.429)	5.309	3.436		0.999		0.0604

Again, three-phase linear was the model which had the worst goodness of fit, with r^2 values as low as 0.92 and RMSE values as high as 0.49.

Table 10 shows average and standard deviation for growth rate of all three microorganisms as obtained by three-phase linear, Gompertz, logistic, and Baranyi models with plate count growth curves. Again, when comparing the similarities in the growth rate values (Table 10), three-phase linear was the model to give less variation (lower standard deviation) for the different growth curves.

Comparisons of the behaviour of different growth models reported in literature have reached different conclusions. Zwietering et al. [3] studied the growth of *Lactobacillus plantarum* in MRS medium at different temperatures and concluded that the Gompertz model was the best-fitting model. When these authors [3] extended the study to several microorganisms they reached similar conclusions. Buchanan et al. [2] reported that the three-phase linear model was more robust than the Gompertz and Baranyi models in terms of successfully fitting growth curve data. In their research [2] they fitted experimental data for *E. coli* O157:H7. Schepers et al. [27] found that the Richards model was the best growth model for *Lactobacillus helveticus* grown at different pH values, and Dalgaard and Koutsoumanis [7] also agreed that Richards model gave the best estimates for absorbance growth curves obtained with mixtures of different microbial strains isolated from spoiled seafood and incubated in different conditions to obtain a wide range of growth yields. López et al. [5] concluded, after a detailed statistical evaluation, that the Baranyi model showed the best behaviour for the growth curves studied. Baranyi and three-phase linear models showed the best fit for plate count data of *Yersinia enterocolitica* grown under different conditions of pH, temperature, and CO_2. Richards model was the best-fitting optical density data of different bacterial and fungal species grown under different conditions. However, Mytilinaios et al. [12] found more recently that Baranyi was the most capable model to fit optical density data obtained for

Table 8: Growth parameters and their 95% confidence limits and coefficients of determination (r^2), SSE, and RMSE of fit obtained with different growth models for average plate count growth curves of *Listeria monocytogenes* CECT 4031 at 37°C in TSB+YE inoculating 10^2, 10^4, and 10^6 CFU mL^{-1} shown in Figure 3(b).

Growth model	Initial concentration (CFU mL^{-1})	Lag time (h)	Growth rate (log cycles h^{-1})	y_0 (log CFU mL^{-1})	C (log cycles)	β	r^2	SSE	RMSE
Three-phase linear	10^2	2.00 (1.85–2.14)	0.487 (0.467–0.506)	1.988 (1.820–2.150)	—		0.997	0.0346	0.0620
	10^4	1.95 (1.75–2.06)	0.432 (0.424–0.441)	4.006 (3.970–4.040)	—		0.986	0.1525	0.1235
	10^6	2.00 (1.78–2.24)	0.423 (0.402–0.441)	6.113 (5.870–6.360)	2.554		0.985	0.0916	0.1070
Gompertz	10^2	2.13 (1.63–2.63)	0.542 (0.510–0.574)	1.803 (1.599–2.007)	6.071 (4.105–8.038)		0.997	0.0406	0.0672
	10^4	2.22 (1.63–2.81)	0.503 (0.443–0.564)	3.932 (3.755–4.109)	3.917 (2.982–4.852)		0.993	0.0691	0.0870
	10^6	2.27 (1.70–2.83)	0.485 (0.417–0.554)	6.009 (5.856–6.163)	3.227 (2.604–3.85)		0.992	0.0655	0.0853
Logistic	10^2	1.73 (0.75–2.71)	0.559 (0.511–0.606)	1.501 (1.086–1.917)	5.297 (3.521–7.073)		0.995	0.0614	0.0826
	10^4	1.91 (0.72–3.10)	0.513 (0.428–0.597)	3.715 (3.340–4.091)	3.739 (2.689–4.79)		0.989	0.1087	0.1099
	10^6	2.12 (1.18–3.06)	0.501 (0.415–0.587)	5.863 (5.599–6.127)	3.073 (2.465–3.681)		0.990	0.0841	0.0967
Richards	10^2	2.13 (1.41–2.85)	0.003 (−2.733–2.738)	1.803 (1.282–2.323)	6.068 (0.701–11.440)	0.002 (−1.865–1.868)	0.996	0.0407	0.0713
	10^4	2.22 (1.57–2.86)	0.0008 (−2.303–2.304)	3.932 (3.614–4.250)	3.917 (2.384–5.450)	0.0006 (−1.686–1.687)	0.992	0.0691	0.0929
	10^6	2.27 (1.70–2.84)	0.013 (0.011–0.015)	6.008 (5.854–6.163)	3.222 (2.601–3.843)	0.01[a]	0.992	0.0657	0.0854
Baranyi	10^2	2.07 (1.84–2.31)	0.514 (0.494–0.534)	1.891	—		0.999		0.0453
	10^4	1.86 (1.34–2.37)	0.447 (0.410–0.483)	3.896	—		0.993		0.0875
	10^6	2.14 (1.64–2.64)	0.460 (0.402–0.517)	5.996	2.774		0.993		0.0774

[a]Value fixed at bound.

L. monocytogenes at different temperatures and pH values. The Weibull model also adequately described microbial growth [5]. Baty and Delignette-Muller [4] found that Baranyi was the best curve-fitting model for most curves, although they noted that the intermodel variability was frequently minor comparing to the imprecision of the parameter estimates, due to the low quantity and quality of the data used to build the growth curves. Actually, the low quality data used by these authors correspond to the datasets tabulated in the work by Buchanan et al. [2]. Pal et al. [28] also showed that Baranyi model provided the best fit for a majority of growth curves obtained for *L. monocytogenes* at low temperatures in liquid cultures, although there was no significant difference among all the primary growth models analysed. Maybe the use of so many microorganisms growing under such different growth conditions could explain, at least in part, the differences in the conclusions reached by authors regarding the best-fitting model.

4. Conclusions

Our results show that both Baranyi and three-phase linear models provide low variability for growth rate values when analysing similar growth curves, hence being the models of choice. Three-phase linear model gives the lowest variation for growth rates, while Baranyi gives a variation marginally higher, despite much better overall fitting.

These results provide insight into predictive microbiology and will help food microbiologists and researchers to choose the proper primary growth predictive model.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Table 9: Growth parameters and their 95% confidence limits and coefficients of determination (r^2), SSE, and RMSE of fit obtained with different growth models for average plate count growth curves of *Escherichia coli* CECT 4031 at 37°C in pH 5 TSB+YE inoculating 10^2, 10^4, and 10^6 CFU mL^{-1} shown in Figure 3(c).

Growth model	Initial concentration (CFU mL^{-1})	Lag time (h)	Growth rate (log cycles h^{-1})	y_0 (log CFU mL^{-1})	C (log cycles)	β	r^2	SSE	RMSE
Three-phase linear	10^2	1.61 (1.41–1.81)	0.518 (0.507–0.526)	2.011 (1.800–2.220)	—		0.992	0.3897	0.1731
	10^4	1.63 (1.39–1.89)	0.521 (0.508–0.544)	4.108 (3.790–4.430)	5.175		0.991	0.4079	0.1771
	10^6	1.74 (1.45–2.02)	0.574 (0.523–0.627)	6.052 (5.920–6.180)	3.229		0.948	0.9450	0.2931
Gompertz	10^2	1.49 (0.57–2.42)	0.576 (0.531–0.620)	1.687 (1.330–2.043)	7.489 (5.845–9.132)		0.997	0.1518	0.1125
	10^4	1.55 (0.97–2.13)	0.580 (0.549–0.611)	3.862 (3.641–4.083)	7.252 (6.270–8.234)		0.999	0.0669	0.0747
	10^6	2.25 (1.63–2.88)	0.692 (0.515–0.869)	6.180 (5.988–6.372)	3.233 (2.898–3.568)		0.985	0.3275	0.1652
Logistic	10^2	0.41 (−1.52–2.34)	0.578 (0.520–0.635)	1.011 (0.223–1.799)	7.501 (5.528–9.474)		0.995	0.2043	0.1305
	10^4	0.85 (−0.11–1.81)	0.593 (0.557–0.630)	3.344 (2.965–3.722)	6.952 (6.051–7.852)		0.998	0.0727	0.0778
	10^6	2.28 (1.64–2.93)	0.728 (0.576–0.880)	6.065 (5.866–6.265)	3.264 (2.987–3.541)		0.992	0.1763	0.1212
Richards	10^2	1.49 (−0.21–3.19)	0.002 (−3.220–3.224)	1.686 (0.453–2.919)	7.488 (4.798–10.180)	0.001 (−2.065–2.067)	0.996	0.1519	0.1175
	10^4	1.53 (0.45–2.60)	0.061 (−1.745–1.867)	3.839 (3.100–4.578)	7.214 (5.605–8.822)	0.041 (−1.248–1.330)	0.998	0.0671	0.0781
	10^6	2.11 (1.08–3.14)	0.953 (0.749–1.157)	5.573 (4.954–6.192)	3.691 (3.069–4.312)	7.363 (−3.876–18.600)	0.996	0.0874	0.0891
Baranyi	10^2	1.32 (0.71–1.92)	0.523 (0.489–0.556)	1.807	—		0.996		0.1176
	10^4	1.79 (1.45–1.92)	0.568 (0.539–0.598)	4.040	5.512		0.999		0.0624
	10^6	2.35 (1.76–2.93)	0.687 (0.528–0.849)	6.181	3.093		0.991		0.1288

Table 10: Average ± standard deviation of growth rate values (log cycles h^{-1}) obtained with three-phase linear, Gompertz, and Baranyi growth models for all the growth curves of *Bacillus cereus* INRA-AVTZ 415 at 30°C in BHI, *Listeria monocytogenes* CECT 4031 at 37°C in TSB+YE, and *Escherichia coli* CECT 515 at 37°C in pH 5 TSB+YE.

Growth model	*B. cereus*	*L. monocytogenes*	*E. coli*
Three-phase linear	1.146 ± 0.035	0.447 ± 0.035	0.538 ± 0.032
Gompertz	1.420 ± 0.164	0.510 ± 0.029	0.616 ± 0.066
Logistic	1.473 ± 0.168	0.524 ± 0.031	0.633 ± 0.083
Baranyi	1.258 ± 0.065	0.474 ± 0.036	0.593 ± 0.085

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Research Article

Twenty Years of *Listeria* in Brazil: Occurrence of *Listeria* Species and *Listeria monocytogenes* Serovars in Food Samples in Brazil between 1990 and 2012

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Listeria spp. isolated from different food products and collected from 12 Brazilian states were sent to the Laboratory of Bacterial Zoonoses (Oswaldo Cruz Institute, Brazil) for identification. The aims of this study were to characterize these isolates, from 1990 to 2012, by using biochemical, morphological, and serotyping tests, and to analyze the distribution of *L. monocytogenes* serotypes on different food products and geographical locations. Serotyping was performed using polyclonal somatic and flagellar antisera. Of 5953 isolates, 5770 were identified as *Listeria* spp., from which 3429 (59.4%) were *L. innocua*, 2248 (38.9%) were *L. monocytogenes*, and 93 (1.6%) were other *Listeria* spp. *L. innocua* was predominantly isolated from 1990 to 2000, while *L. monocytogenes* was from 2001 to 2012. Regarding the serotype distribution in the foods, serotypes 1/2a and 4b were most common in processed meat and ready-to-eat products, respectively; serotypes 1/2a, 1/2b, and 4b were the most common in nonprocessed meat. The results above confirm the presence of the main serotypes of *L. monocytogenes* in different parts of the food chain from three regions of the country and emphasize the importance of improving the control measures, as tolerance zero policy and microbiological surveillance in Brazil.

1. Introduction

The genus *Listeria* includes pathogenic species as *Listeria monocytogenes* and *Listeria ivanovii,* the latter is common in warm-blooded animals, and it is widespread in nature. Its ubiquity is evident by the isolation of this microorganism in fecal specimens from healthy hosts, soil, water, waste, vegetables, and processed silage or food (as well as their factories) [1].

Listeria monocytogenes differs from most bacterial food pathogens due its ability to survive harsh environment conditions, grow over a wide temperature range (1–45°C), and survive under wide pH range (4.5–9.6), high salt concentration (10 to 15% NaCl), and very low water activity (aw = 0.94). Therefore it can grow in different types of food products [2].

From a public health perspective, *L. monocytogenes* (mainly the serotypes 1/2a, 1/2b, and 4b) is responsible for severe syndromes in humans, such as meningitis, septicemia, abortion, and febrile enteritis. Its main impact is in immunocompromised individuals, with a high fatality rate of 20% to 30% [3]. As it is a foodborne pathogen, individuals are infected predominantly through contaminated food consumption [4].

Although sporadic cases of listeriosis have been reported in Brazil [5–8], there is no information on foodborne outbreaks involving *L. monocytogenes* [9, 10]. On the other hand, different foods have been recognized as potential sources of this pathogen [6–8, 11–18], but there is no systematic evaluation of these products over time.

In Brazil the Normative Ruling Number 09, promulgated on 8 April 2009 by the Ministry of Agriculture, Cattle Raising and Supply, established criteria and procedures for the implementation of *L. monocytogenes* Control Procedures in ready-to-eat products of animal origin in order to monitor and ensure the safety of these products. These procedures are supervised by a national program called Federal Inspection Service (SIF). The National Agency of Sanitary Surveillance also has the RDC (Board Resolution) Number 12 on 2 January 2001, which establishes the absence of this pathogen in 25 g sample of cheese [19, 20].

Since there is no compulsory notification for cases of Listeriosis in Brazil, Sanitary Surveillance and Public Health has trouble to identify the occurrence of outbreaks, and only isolated cases are reported, which may explain the few listeriosis cases in the literature and the absence of outbreaks reports in our country.

The aims of this study were to (i) identify the species and serotypes of *Listeria* isolated from different food products and regions of Brazil and (ii) analyze the presence of main serotypes of *L. monocytogenes* in different foods and geographical locations of Brazil in an attempt to present a more detailed view of the distribution of *Listeria* spp. in the country.

These isolates were received by the Laboratory of Bacterial Zoonoses (LABZOO) at Oswaldo Cruz Institute (IOC), FIOCRUZ, Rio de Janeiro, Brazil, for identification and serotyping.

2. Materials and Methods

2.1. Bacterial Cultures. From 1990 to 2012 the laboratory received, from different sources (industries, public universities, research institutes, and other public institutions), 5953 isolates suspected of being *Listeria* spp. for identification, isolated from several food products of 12 different Brazilian states. All these isolates were received in nutrient agar.

2.2. Identification of Listeria spp. All isolates were screened through hemolysis and colony morphology on Columbia agar containing 5% defibrinated sheep blood, evaluation of the mobility to 25–28°C by stab-inoculation in semisolid agar (tryptose broth containing 0,4% agar), and Gram staining. In this initial screening, 183 (3.07%) isolates were discarded which includes 156 nonmotile Gram positive cocci and 27 motile Gram negative rods. Consequently, 5770 (96.9%) isolates went to further identification and serotyping. The identification was performed using previously published morphological and biochemical tests characterized by catalase, motility, and biochemical tests including acid production from D-xylose, D-mannitol, L-rhamnose, and α-methyl-D-mannoside. When necessary, *L. monocytogenes* was differentiated from other species of *Listeria* by using API *Listeria* (BioMeriéux) kit. The final confirmation was provided by Christie-Atkins-Munch-Peterson (CAMP) Test [21].

All isolates identified as *Listeria* were stored in Brain Heart Infusion (Difco) with 20% (v/v) glycerol at −80°C. All the *Listeria* isolates were deposited in the Culture Collection of *Listeria* (CLIST/LABZOO).

2.3. Listeria spp. Serotyping. The isolates were serotyped using polyclonal antisera produced against *Listeria* somatic and flagellar antigens previously manufactured in the LABZOO according to the method described by Seeliger and Höhne [22].

2.4. Statistical Analysis. All the isolates were tabulated using Excel 2007 software. Subsequently, the distribution of all continuous variables, means, medians, and interquartile values were studied. The frequency of all the categorical variables was described. Bivariate analysis of continuous variables was performed using Student's t-test or Mann-Whitney test if the variable did not follow normal distribution. Bivariate analysis of categorical variables was performed using Fisher exact test. Statistical analysis was performed using the statistical package STATA version 13.0, Texas, USA.

3. Results

The sources of *Listeria* isolates sent to the lab are geographically depicted in Table 1. Of those, 4714 (81.7%) were meat products (processed or/and not processed). Most of the strains (1474, 31.27%) were from poultry products and originated from Goiás, a state in the Middle-West of Brazil.

A total of 1404 (24.3%) strains were isolated from processed meat products (cooked, cured, or smoked products), with the most frequent (900 strains) being mixed sausages (chicken and pork) from two Southeastern Brazilian states. As for dairy products, the home-made cheese from the Southern Brazilian state of Rio Grande do Sul was the main origin.

As can be seen in Table 1, there was a predominance of the southeastern region (states of Minas Gerais, Rio de Janeiro, and São Paulo), with 2711 strains (46.9%), followed by the Midwestern region (states of Mato Grosso and Goiás) with 1817 strains (31.4%), mainly from meat products. These two regions contributed a total of 4528 strains of *Listeria* (78.3%). On a smaller scale, the South and Northeast regions totalized 1078 (18.6%) and 164 strains (2.82%), respectively, mainly from meat and dairy products. Among the states with isolated *Listeria* spp. in dairy products, São Paulo and Rio Grande do Sul contributed with more strains. While São Paulo had a similar number of *Listeria* strains from milk and cheese, in Rio Grande do Sul cheese was the major source (OR = 8.8, 95% CI = 5.2–14.9, $p < 0.01$).

The characterization of the species in the period of 1990–2012 (Figure 1) revealed the prevalence of *L. innocua* (3429, 59.4%) and *L. monocytogenes* (2248, 38.9%) and discrete occurrences of *L. welshimeri*, *L. seeligeri,* and *L. grayi* subsp. *murrayi* (93, 1.6%). However, *L. ivanovii* was not isolated from any of the food materials.

Table 1: Geographical distribution of *Listeria* spp. strains by food type in Brazil. 1990–2012.

State	Region*	Milk	Cheese	Meat		Ready-to-eat	Vegetables	Unknow source**	Total	
				Nonprocessed	Processed				*N*	%
Alagoas	NE		34						34	0.58
Bahia	NE	4	1	39	19	2			65	1.12
Paraíba	NE	25	8						33	0.57
Pernambuco	NE		28	2	2				32	0.55
Goiás	MW			1474	60				1534	26.58
Mato Grosso	MW		17	43	223				283	4.9
Minas Gerais	SE		87	346	472		17		922	15.97
Rio de Janeiro	SE	2	36	474	57	8	26		603	10.45
São Paulo	SE	92	95	352	428	114	56	49	1186	20.55
Paraná	S		12	29	4				45	0.77
Santa Catarina	S		42	380	4	79			505	8.75
Rio Grande do Sul	S	22	200	171	135				528	9.15
Total		145	560	3310	1404	203	99	49	5770	

*NE, Northeast; MW, Middle-West; SE, Southeast; S, South.
**Food source not informed by the senders.

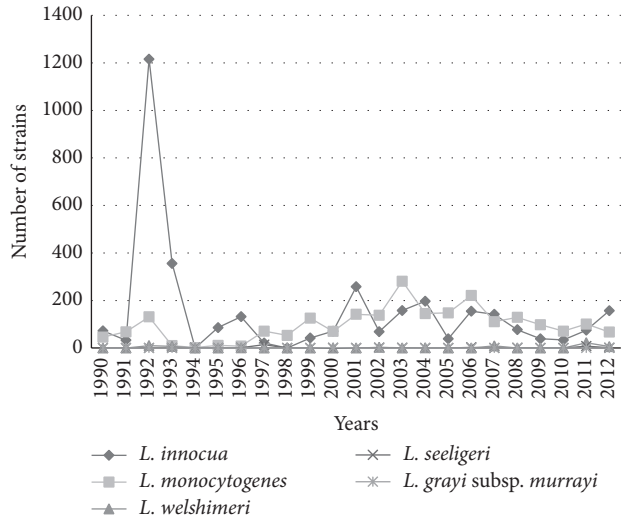

Figure 1: Temporal distribution of *Listeria* spp. isolated from food in Brazil from 1990 to 2012.

The analysis of the trend isolation of *L. monocytogenes* and *L. innocua* during two periods, 1990–2000 and 2001–2012, (number of strains in the period/total strains for the species) showed that *L. innocua* in the first period amounted to 58.9% (2029/3429) and to 40.8% (1400/3429) in the second period. As for *L. monocytogenes*, it amounted to 26.5% (596/2248) in the first period and reached 73.4% (1652/2248) in the second (OR = 3.92, 95% CI = 3.50–4.39, $p < 0.01$).

In species distribution by geographical origin (Table 2), four states, Paraíba, Mato Grosso, São Paulo, and Rio Grande do Sul, had higher levels of *L. monocytogenes* strains (1456 of 2030 strains, 71.7%), surpassing the other *Listeria* spp. isolated, while this was not observed in the other states (792 of 3740 strains, 21.2%) (OR = 9.4, 95% CI = 8.3–10.7, $p < 0.01$). In contrast, in the other states, the prevalence of *L. innocua* (2876 of 3740 strains, 76.9%) was higher (OR = 8.9, 95% CI = 8.9–10.1, $p < 0.01$).

Based on the regional distribution of the species, of the 4692 strains from Northeast, Midwest, and Southeast regions, 2954 (62.9%) were identified as *L. innocua*, while 1684 (35.8%) were identified as *L. monocytogenes*. In the South region, with 1078 strains, *L. monocytogenes* was more frequent (564-52.3%) than *L. innocua* (475-44%) (OR = 2.1, 95% CI = 1.8–2.4, $p < 0.01$), a result biased by the sampling from Rio Grande do Sul.

Regarding the distribution of *L. monocytogenes* serotypes in food products, Table 3 shows that those referred to as the most potentially pathogenic isolates to human consumers, 1/2a, 1/2b, and 4b, were more prominent at 30.3% (1751/5770), rising to 36.8% when serotype 1/2c samples (2129/5770) were included in the calculation. Moreover, the predominance of serotype 4b on most products is evident, except in processed meat, where serotype 1/2a was predominant. When we considered all products, the serotypes 1/2a and 4b presented a similar contribution (10,97% and 11,29%, resp.).

The serotypes 3a, 3b, 3c, 4c, 4e, and 7 and nontypable samples, with rough R-forms, reached a minimum level (119–2.06%), and the isolation of the serotype 4e in milk samples from São Paulo and unprocessed meats (beef and chicken) from Rio Grande do Sul is also noteworthy.

L. innocua constituted the largest number of strains (3429 of 5770 strains, 59.4%), of which 2943 were identified as serotype 6a, isolated mainly in unprocessed (poultry) and processed meat (sausages), totaling 2554 of 2943 strains (86,8%).

4. Discussion

In the period of 1990–2000, several human listeriosis outbreaks were described in the world with food products

Table 2: Geographical distribution of *Listeria* spp. isolates, in Brazil. 1990–2012.

States	Species					Total	
	L. innocua	*L. monocytogenes*	*L. welshimeri*	*L. seeligeri*	*L. grayi* subsp. *murrayi*	*N*	%
Alagoas	22	12				34	0.58
Bahia	57	6			2	65	1.12
Paraíba	6	27				33	0.57
Pernambuco	28	4				32	0.55
Goiás	1350	169	15			1534	26.58
Mato Grosso	64	216		3		283	4.9
Minas Gerais	763	154	3	2		922	15.97
Rio de Janeiro	330	259		14		603	10.45
São Paulo	334	837	13	2		1186	20.55
Paraná	28	14	3			45	0.77
Santa Catarina	298	174	25	8		505	8.75
Rio Grande do Sul	149	376		3		528	9.15
Total							
N	3429	2248	59	32	2	5770	99.8
%	59.42	38.96	1.02	0.55	0.03		

as their source; consequently, an intense policy with the implementation of control measures in food industries by Hazard Analysis and Critical Control Points (HACCP) and risk analysis were adopted mainly in Europe and North America. In addition, there are specific recommendations for persons at higher risk for listeriosis. There was also an effort to strengthen quantitative microbiological criteria, with a zero tolerance policy for foods that support and favor the multiplication of bacteria [3, 23]. It should be noted that the policy of zero tolerance has been criticized on the basis that the level of contamination by *L. monocytogenes* in marketed products is often very low and in some developed countries this measure revealed no significant change on listeriosis incidents [24].

In the present study, the laboratory received suspected isolates of *Listeria* spp. However, the sender did not inform if they had established a standard microbiological acceptability of the product or product batches based on it or the presence or number of bacterial masses per unit area or batch. There was also no information about where the products came from, if they were from other geographical areas of Brazil, or if they were imported or would be exported.

The data in Table 1 shows how common members of the genus *Listeria* isolated from foods are in the four regions of Brazil. The lack of samples from the North of Brazil does not indicate that this microorganism is not present in this area. In fact, [25] reported the isolation of *Listeria* spp. in beef in the city of Belém, state of Pará (one of the main cities from Northern Brazil).

Quantitative variations occurred mainly due to the higher concentration of industries that processed food products of animal origin, in particular meat products, mainly from Middle-West, Southeast, and South regions and these are sold to domestic and international markets. On a smaller scale, but present in all regions examined, are dairy products; their marketing is more restricted to the area surrounding the manufacturing site and many products are identified as artisanal.

From an epidemiological point of view, the movement of food carriers is probably an important route for *L. monocytogenes* spread to domestic consumers and those from other regions and/or countries [26].

As for the temporal distribution of isolates characterized into species in the two periods (1990–2000 and 2001–2012) (Figure 1), *L. innocua* had marked predominance in the first period when compared to *L. monocytogenes*. In the second period, the opposite was seen, and we hypothesized that a possible cause for the difference was the improvement of isolation technique by the senders of the samples, as a result of implemented methods for analysis of foods such as the introduction of new selective culture media [27, 28]. Another important detail was that the technical guidance stating that a number of 5 or more suspected isolated colonies in a selective media should be screened for *Listeria* spp. decreased the number of false-positives. In the confirmatory phenotypic analysis, the hemolysis test would frequently yield false-negative results, and this is a critical test in the differentiation between *L. monocytogenes* and *L. innocua*. To overcome this problem, the CAMP test, the alanyl peptidase detection, or molecular typing [28] is recommended.

As for the other species (Figure 1 and Table 2), there was a low incidence of *L. welshimeri, L. seeligeri,* and *L. grayi* subsp. *murrayi* (93 strains, 1.6%) and absence of *L. ivanovii* isolates, according to the findings of Gianfranceschi et al., 2003 [29]. In Table 2 it was observed that four states located in each region revealed the prevalence of *L. monocytogenes*, in opposition to the other areas where *L. innocua* had a higher incidence. Analyzing the results, it can be assumed that there exists a correlation with the type of product, especially meat products (processed and unprocessed) of

Table 3: Species and serovar frequency of *Listeria* spp. isolates from food, in Brazil. Period 1990–2012.

Food type	*L. innocua*			*L. monocytogenes*											*L. welshimeri*	*L. seeligeri*	*L. grayi*	Total	
	6a	6b	NT*	1/2a	1/2b	1/2c	3a	3b	3c	4b	4c	4e	7	NT*	R**				*N*	%
Meat:																				
Nonprocessed	1939	90	233	244	250	140	6	20	2	275		28	1	2	4	51	25		3310	57.37
Processed	615	53	27	239	113	144	5	8		163	2	4		18		8	5		1404	24.33
Ready-to-eat	387	38	45	139	90	80		1		195		13	1	4			2	2	997	17.28
Unknown	2			11	13	14				19									59	1.02
Total																				
N	2943	181	305	633	466	378	11	29	2	652	2	45	2	24	4	59	32	2	5770	100
%	51.0	3.14	5.29	10.97	8.08	6.55	0.19	0.51	0.04	11.29	0.04	0.78	0.04	0.41	0.06	1.02	0.55	0.04		

*NT = nontypable (smooth form).
**R = rough form.

avian origin, particularly composed of poultry meat sausages. This situation is depicted in isolates from Bahia, Goiás, Minas Gerais, Rio de Janeiro, and Santa Catarina. It was observed that in isolates from unprocessed meat products (cattle, sheep, and pigs) the occurrence of *L. innocua* was not as relevant and in some situations *L. monocytogenes* had a higher incidence in meat products from avian sources in states of Mato Grosso, São Paulo, and Rio Grande do Sul. A hypothesis for the variations in prevalence of the species, whatever the food analyzed is, is that *L. innocua*, when present in equal or greater numbers, tends to overgrow *L. monocytogenes* during the stages of selective enrichment, resulting in a smaller number of *L. monocytogenes* colonies and greater difficulty in viewing them in isolation medium [28].

The presence of *L. monocytogenes* serotypes isolated from various types of food (Table 3) reproduces previous observations from both national [6–8, 11, 16, 17] and international investigations [3, 21, 30] demonstrating the predominant and cosmopolitan nature of the 1/2a, 1/2b, and 4b serotypes, but with increased detection of serotype 1/2c. Overall, these serotypes were found in all products analyzed, with serotype 4b isolates prevailing in ready-to-eat products, serotype 1/2a prevailing in processed meat, and a similar distribution of the serotypes 1/2a, 1/2b, and 4b in nonprocessed meat.

From an epidemiological standpoint, serotyping has a very limited discriminatory level, and only three serotypes (1/2a, 1/2b, and 4b) have a predominant role in human disease processes [31], although presumably serotyping could allow investigators to understand changes in the temporal occurrence of serotypes by geographical region and a particular food type. Nevertheless, serotyping information is necessary to establish the association of serotypes and outbreaks [32].

The observations of Parihar et al. [32] are supported by numerous references about the predominance of serotype 1/2a in clinical cases in Sweden, perhaps as a result of its predominance in many foods, including processed foods. This situation was also reported in other European countries and in the U.S., with serotype 1/2a isolates from food and clinical cases reported more frequently than 4b [23], in gastrointestinal listeriosis [3], or non-outbreak-associated cases of *L. monocytogenes* infection [28]. In our research, the difference between the two serotypes (4b with 652 isolates and 1/2a with 633 isolates) is not significant, even when the isolates related to food without identification are not considered. The frequency of serotypes 1/2b and 1/2c was consistent with previous findings with meat products as the main source of isolation [6, 8, 29]. The antigenic characterization of the predominant species in isolates, *L. innocua*, demonstrated the clear predominance of serotype 6a, mainly in isolates from unprocessed meat products (poultry) and processed meat (sausages containing beef and poultry) as well as in cheeses. The prevalence of other species, identified as *L. welshimeri, L. seeligeri,* and *L. grayi* subsp. *murrayi*, was very low; they were mainly found in nonprocessed meat products.

The association of the resistance of *Listeria* spp. to the conditions imposed by the environment and its wide dissemination in animal sources is a serious problem for the food industry. This situation is a result of the high level of contamination of raw materials originating from animals and vegetables, as these microorganisms may persist for variable periods in the industrial environment. Of particular importance is the ability of *L. monocytogenes* to adhere to surfaces and form biofilm [33]. In the food markets and home environments, a flaw in the cold chain constitutes a risk factor for the growth and dissemination of *Listeria* [3, 34].

The situation depicted by this study demonstrates how easy the transmission of *L. monocytogenes* to the consumer is and the underlying reasons for sporadic cases and outbreaks of listeriosis [3, 35]. Indeed, individuals affected by *Listeria monocytogenes* must encounter the conditions recognized as risk factors [36]. Based on this, attention should be focused on the analysis of risk assessments for ready-to-eat foods, including the establishment of quantitative microbiological criteria as adopted in the U.S. and Europe [3, 35, 37, 38].

Given the results related to ready-to-eat products (Table 3), there is no doubt that consumers are exposed to potential risks of infection, especially the most susceptible population: immune suppressed patients, individuals on extreme age groups, and pregnant women. Furthermore, it is important to take into account the ingested dose of viable bacteria, in addition to the characteristics of the host. This is influenced by the period that the products are kept under refrigeration and the ability of the bacteria to grow in these products [37]. The adoption of a program to reduce the salt concentration in food products (to reduce hypertension) in France raised the hypothesis that this public health measure may have increased the chance of *L. monocytogenes* contamination in foods (mostly meat and fish) and increased the disease incidence [38, 39].

Although the results presented here point to the presence of the main serovars of *L. monocytogenes* in different foods, there are no reports of outbreaks of listeriosis in the country, only a few sporadic cases. This fact can be explained by the absence of mandatory reporting in cases of listeriosis, since there is no compulsory notification for cases of listeriosis in Brazil, and also brings up the possibility that those responsible for the diagnosis do not have proper training to identify this pathogen.

In summary, our work depicts a current overview of the genus *Listeria* distribution in primary foods used as raw material in large national food industry that remain in the final product even after processing.

The eradication of *L. monocytogenes* in foods and processing environments is a difficult task due to the ability of this pathogen to adapt to different harsh conditions. Therefore, it is fundamental the application of HACCP and GMP (Good Manufacturing Practices) programs in industries, a stronger microbiological surveillance and greater dissemination of information about listeriosis (especially for risk groups). Furthermore a network bringing together the public health and food surveillance is necessary in order to gather more efforts to monitor and reduce the risks of food contamination by *L. monocytogenes* in Brazil.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Surveillance of Food- and Smear-Transmitted Pathogens in European Soldiers with Diarrhea on Deployment in the Tropics: Experience from the European Union Training Mission (EUTM) Mali

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Introduction. Since 2013, European soldiers have been deployed on the European Union Training Mission (EUTM) in Mali. From the beginning, diarrhea has been among the most "urgent" concerns. Diarrhea surveillance based on deployable real-time PCR equipment was conducted between December 2013 and August 2014. *Material and Methods*. In total, 53 stool samples were obtained from 51 soldiers with acute diarrhea. Multiplex PCR panels comprised enteroinvasive bacteria, diarrhea-associated *Escherichia coli* (EPEC, ETEC, EAEC, and EIEC), enteropathogenic viruses, and protozoa. Noroviruses were characterized by sequencing. Cultural screening for Enterobacteriaceae with extended-spectrum beta-lactamases (ESBL) with subsequent repetitive sequence-based PCR (rep-PCR) typing was performed. Clinical information was assessed. *Results*. Positive PCR results for diarrhea-associated pathogens were detected in 43/53 samples, comprising EPEC ($n = 21$), ETEC ($n = 19$), EAEC ($n = 15$), Norovirus ($n = 10$), *Shigella* spp./EIEC ($n = 6$), *Cryptosporidium parvum* ($n = 3$), *Giardia duodenalis* ($n = 2$), *Salmonella* spp. ($n = 1$), Astrovirus ($n = 1$), Rotavirus ($n = 1$), and Sapovirus ($n = 1$). ESBL-positive Enterobacteriaceae were grown from 13 out of 48 samples. Simultaneous infections with several enteropathogenic agents were observed in 23 instances. Symptoms were mild to moderate. There were hints of autochthonous transmission. *Conclusions*. Multiplex real-time PCR proved to be suitable for diarrhea surveillance on deployment. Etiological attribution is challenging in cases of detection of multiple pathogens.

1. Introduction

Diarrhea due to food- and smear-transmitted pathogens remains a scourge of military deployments in tropical settings. Potential deleterious consequences of this problem were first described more than 3,000 years ago in the Old Testament (Deuteronomy 23: 9–14), detailing basic hygiene procedures such as isolation of infected soldiers and eradication of infectious material in times when there was no concept

of microbial pathogens. Recently described deployment-associated outbreaks of gastrointestinal infections confirm the relevance of the issue [1, 2].

As previously described [3], infections with food- or waterborne enteric pathogens can be efficiently prevented by elaborate hygiene precautions in military field camps on deployment. In German field camps, these precautions comprise compliance with European general principles and requirements of food law, European procedures regarding food safety (Regulation EC number 178/2002), and the German Food and Feed Law ("Lebensmittel- und Futtermittelgesetzbuch," LFBG); production and delivery of food and drinking water by German soldiers or under their direct supervision whenever possible; implementation of HACCP (hazard analysis and critical control points) systems from delivery to disposal, including cleaning and disinfection measures in conjunction with food production as well as handling procedures by operators of dining and water treatment facilities; and instructions in accordance with the German infection prevention law ("Infektionsschutzgesetz," IfSG) for military and civilian staffs of facilities supplying food and water. Military public health officials such as veterinarians or hygiene officers are in charge of all food and drinking water control procedures. Laboratory surveillance, generally focused on infectious and noninfectious threats that might endanger the mission, is carried out with samples of delivered and prepared food and treated water prior to release and also includes the screening of local staffs for pathogens according to relevant directives from the hygiene department. Generally, all deployed soldiers are by order forbidden to consume other than safety-approved food and drinking water from the country of deployment. Such safety approval requires that local producers are audited and controlled by military food specialists.

If such high hygiene standards can be maintained, infection rates with enteric pathogens are comparably low to those in Germany as shown for chronic infestations with enteropathogenic protozoa in German soldiers returning from German military field camps in various subtropical and tropical deployment sites [3]. If these standards cannot be maintained for logistic reasons, for example, during small or multinational deployments, infection rates increase [3]. Further, in spite of training and education in basic hygienic measures that are implemented before deployment, soldiers may tend to noncompliance if the temptation of appealing local foods coincides with monotonous food in the field canteen or perhaps delivery problems during the first phases of deployment. A previous study described noncompliance problems with antimalarial chemoprophylaxis on deployment [4].

German hygiene standards for military field camps cannot always be maintained during multinational military operations. German soldiers have, for example, participated in the European Union Training Mission (EUTM) in Mali since March 2013, with the field camp in Koulikoro near the capital Bamako as the major site of deployment. Diarrhea has been among the most "urgent" infectious concerns from the beginning. Consequently, deployable real-time PCR equipment was transferred to Koulikoro to study the molecular epidemiology of diarrhea in the camp from December 2013 until March 2014. Subsequently, the surveillance of food- and smear-transmitted pathogens was continued until August 2014 by transferring sample material to Germany. Here we describe results that were obtained in a 9-month observation period.

2. Materials and Methods

2.1. Study Population. Between the 49th calendar week in December 2013 and the 34th calendar week in August 2014, microbiological surveillance was carried out on European soldiers with acute diarrhea deployed in Mali in the course of the European Union Training Mission (EUTM). Diarrhea was defined as the deposition by the subject of amorphous stools and distinguished according to frequency as <3 or ≥ 3 stools per day. Accordingly, low-frequency diarrhea was considered as of potential infectiological relevance as well. The field doctor in charge subjectively decided whether or not he or she considered the symptoms of the patients to be relevant and incapacitating enough for an inclusion into the surveillance. All diarrhea patients were asked by the field doctor in camp Koulikoro to provide a sample of native stool for molecular and cultural diagnostic analysis. Investigations of diseased local military or civilian personnel were not part of the surveillance.

In addition to providing the samples, the field doctor collected data on diarrhea patients in a standardized way on the delivery note that was sent together with the sample. The items on these notes were as follows: age, gender, nationality, mode of food intake, site of deployment, stool frequency, stool consistency as described by the patient, accompanying symptoms, presence of fever, type of antimalarial prophylaxis, vaccination status against cholera and typhoid fever, medication with antibiotic drugs, any previous stays in high-endemicity settings regarding diarrhea (including Afghanistan, Bahrain, Bolivia, Colombia, Kosovo, Lebanon, and Turkey [3]), and participation in counseling regarding hygiene on tropical deployments prior to the onset of diarrhea. In addition, stool consistency of the samples provided was assessed by the analyzing laboratory technician.

While providing stool samples and thus participating in the surveillance were voluntary, all cases of diarrhea among EUTM personnel that were registered by the field doctor were further routinely reported to the Deployment Health Surveillance Capability (DHSC) of the North Atlantic Treaty Organization (NATO) Military Medicine Center of Excellence. Again, the diagnosis diarrhea was based on the field doctor's subjective decision whether or not he or she considered the symptoms of the patients to be relevant and incapacitating. These data and the number of deployed EUTM forces were used to calculate a weekly incidence rate as a passive surveillance procedure. DHSC reports were compared with the number of patients that were included in the surveillance to estimate the achieved coverage.

2.2. Laboratory Testing. From December 2013 to March 2014, collected stool samples were analyzed by PCR directly in the

laboratory of the field hospital in Camp Koulikoro in Mali. From April until August 2014, the samples were frozen at −20°C and shipped to Germany for further investigation. All culture-based diagnostic approaches were performed in Germany.

2.2.1. Sample Preparation. Prior to PCR analysis, nucleic acids were extracted from all stool samples using the Qiamp stool kit (Qiagen, Hamburg, Germany). The nucleic acid extraction was performed as described by the manufacturer.

2.2.2. PCR Testing. All samples were analyzed with a panel of real-time multiplex PCRs comprising two in-house protocols. One of those in-house PCR tests targets the invasive enteropathogenic bacteria *Salmonella* spp., *Shigella* spp./enteroinvasive *Escherichia coli* (EIEC), *Campylobacter jejuni*, and *Yersinia* spp. [5, 6]. The diagnostic reliability of this procedure has been shown previously in comparison with cultural approaches [5]. The other in-house PCR, which is used for routine diagnostic analyses by the German National Reference Centre for Tropical Diseases Bernhard Nocht Institute Hamburg, amplifies DNA of the enteropathogenic protozoa *Entamoeba histolytica*, *Giardia duodenalis*, *Cyclospora cayetanensis*, and *Cryptosporidium parvum* [3, 6, 7]. The previously described primer-probe-sets for *Entamoeba histolytica*, *Giardia duodenalis*, and *Cryptosporidium parvum* were complemented by a primer-probe-set for *Cyclospora cayetanensis* that was adapted from a previous publication [7]. No further changes to the described protocols [3, 5, 6, 8] were applied. The primer-probe-sets used are detailed in Table 1.

In addition to the in-house multiplex real-time PCR assays described above, three commercial RidaGene (R-Biopharm, Darmstadt, Germany) PCR kits: "EAEC," "EHEC-EPEC," and "ETEC-EIEC," were applied targeting enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxic *E. coli* (ETEC), and *Shigella* spp./EIEC. Finally, analyses using the commercial Fast-track Diagnostics (Sliema, Malta) PCR kit "viral gastroenteritis" targeting Norovirus genogroups I (G1) and II (G2), Astrovirus, Rotavirus, Adenovirus, and Sapovirus were added.

Phocid herpesvirus DNA was chosen as the target for inhibition control PCR. The procedure was performed as described previously [8–10]. The primer-probe-set is presented in Table 1.

2.2.3. Norovirus Sequencing. Samples testing positive by Norovirus genogroup I (GI) or II (GII) PCR were subjected to further sequence analysis. In detail, a 213-base-pair (bp) part of the open reading frame 1 (ORF1) was amplified using degenerate multiplex primers MON432/MON434 (GI) and MON431/MON433 (GII) as described [11]. Amplified DNA was sent for sequencing (Seqlab, Göttingen, Germany) after nucleic acid gel extraction and cleanup with a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Sequence assembly and analysis were performed with the software DNAStar Lasergene 12.1 genomics suite (DNASTAR Inc., Madison, WI, USA). Subsequent genotyping was based upon the Norovirus genotyping tool as described [12].

2.2.4. Cultural Growth, Identification, and Resistance Testing. If sufficient stool material was available, cherry-pit-sized volumes were used for broth enrichment in thioglycolate broth (Heipha, Eppelheim, Germany). This was the case for a total of 48 out of 53 samples. Incubation was performed for 16–24 hours at 37°C. Broth enrichment increases the yield of ESBL-expressing bacteria after swabbing, for example, by a factor of 2 in upper respiratory tract samples [13]. Subsequently, 10 μL preincubated broth was cultured on Brilliance ESBL selective agar (Oxoid, Basingstoke, UK). This agar is made for selective growth of ESBL-positive Enterobacteriaceae. Sensitivity of 94.9–97.9% and specificity of 95.7–100% have been described for Brilliance ESBL selective agar [14, 15]. Agar plates were incubated at 37°C for 40–48 hours. All colonies that looked suspicious for Enterobacteriaceae (blue, green, and brown colonies) were isolated, while suspected Gram-negative nonfermentative rod-shaped bacteria (i.e., yellow or yellowish-brown or greenish-brown colonies) were discarded. All isolates were frozen at −80°C in Microbank tubes (Pro-Lab Diagnostics, Bromborough, UK) until further assessment.

Identification was performed by VITEK-II GN-cards (bioMérieux, Marcy-l'Étoile, France) and matrix-assisted laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using a Shimadzu/Kratos "AXIMA Assurance" MALDI-TOF mass spectrometer (Shimadzu Germany Ltd., Duisburg, Germany) [16]. For MALDI-TOF analyses, isolates were prepared using alpha-cyano-4-hydroxycinnamic acid (bioMérieux) as matrix. Spectral fingerprints were analyzed using Vitek MS IVD V2, database MS-CE version CLI 2.0.0 (bioMérieux). Automated antibiotic susceptibility testing was performed with VITEK-II AST-N263-cards (bioMérieux). In case of uncertain results, E-testing (bioMérieux) was added. Interpretation of resistance testing results was based on the EUCAST guideline (version 4.0, 2014, http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.pdf).

2.2.5. Rep-PCR Typing of Extended-Spectrum Beta-Lactamase Positive E. coli. All confirmed ESBL-positive *E. coli* strains from ESBL selective agar were grown overnight in brain heart infusion broth. DNA was extracted using the MoBio UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). Purified DNA samples were amplified using the DiversiLab *Escherichia* DNA fingerprinting kit (reference number: 410 980, bioMérieux) on a T-personal thermal cycler (Biometra, Göttingen, Germany). Rep-PCR products were detected by chip-based DNA separation on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). All techniques were executed according to the manufacturers' instructions.

Documentation and band-pattern analysis were performed using the DiversiLab software version 3.3 (bioMérieux). A correlation cutoff of 95% for confirmation or exclusion of clonal identity of analyzed strains was applied as recommended by the manufacturer. All library entries were analyzed in duplicate.

Table 1: Sequences of the primer-probe-sets used in the applied real-time PCR assays [5–9].

Target organism	Forward primer	Reverse primer	TaqMan probe
Multiplex-PCR targeting invasive enteric bacteria			
Salmonella spp.	5′-ATT-GTT-GAT-TCA-GGT-ACA-AAC-3′	5′-AAT-TAG-CCA-TGT-TGT-AAT-CTC-3′	5′-CAA-GTT-CAA-CGC-GCA-ATT-TA-3′
Shigella spp./EIEC	5′-CAG-AAG-AGC-AGA-AGT-ATG-AG-3′	5′-CAG-TAC-CTC-GTC-AGT-CAG-3′	5′-ACA-GGT-GAT-GCG-TGA-GAC-TG-3′
Campylobacter jejuni	5′-CTA-TAA-CAA-CTG-CAC-CTA-CTA-AT-3′	5′-AAG-TGT-AAG-CAC-ACA-AGG-TA-3′	5′-CTT-AAT-AGC-CGT-CAC-CCC-AC-3′
Yersinia spp.	5′-GCA-TTA-ACG-AAT-ATG-TTA-GC-3′	5′-ATC-GAG-TTT-GGA-GTA-TTC-AT-3′	5′-CCG-CTT-CCA-AAT-TTT-GTC-AT-3′
Multiplex-PCR targeting enteric protozoa			
Entamoeba histolytica	5′-ATT-GTC-GTG-GCA-TCC-TAA-CTC-A-3′	5′-GCG-GAC-GGC-TCA-TTA-TAA-CA-3′	5′-TCA-TTG-AAT-GAA-TTG-GCC-ATT-T-3′
Giardia duodenalis	5′-GAC-GGC-TCA-GGA-CAA-CGG-TT-3′	5′-TTG-CCA-GCG-GTG-TCC-G-3′	5′-CCC-GCG-GCG-GTC-CCT-GCT-AG-3′
Cyclospora cayetanensis	5′-TAG-TAA-CCG-AAC-GGA-TCG-CAT-T-3′	5′-AAT-GCC-ACG-GTA-GGC-CAA-TA-3′	5′-CCG-GCG-ATA-GAT-CAT-TCA-AGT-TTC-TGA-CC-3′
Cryptosporidium parvum	5′-CGC-TTC-TCT-AGC-CTT-TCA-TGA-3′	5′-CTT-CAC-GTG-TGT-TTG-CCA-AT-3′	5′-CCA-ATC-ACA-GAA-TCA-TCA-GAA-TCG-ACT-GGT-ATC-3′
Internal control PCR targeting phocid herpesvirus DNA			
Phocid herpes virus	5′-GGG-CGA-ATC-ACA-GAT-TGA-ATC-3′	5′-GCG-GTT-CCA-AAC-GTA-CCA-A-3′	5′-TTT-TTA-TGT-GTC-CGC-CAC-CAT-CTG-GATC-3′

Table 2: Median and mean cycle threshold (Ct) values of pathogens that were detected by PCR.

Detected pathogen	Median Ct-value	Mean Ct-value	Standard deviation
Salmonella spp.	27	27	—
Shigella spp./EIEC[1]	17.5	18	3.0
Shigella spp./EIEC[2]	13.5	14.7	2.7
Shigella spp./EIEC[3]	12.5	13.7	2.4
EPEC	22	21.4	4.0
STEC[4]	14	15.9	3.9
STEC[5]	19	18.2	4.3
EAEC	19	19.2	3.5
Giardia duodenalis	25.5	25.5	3.5
Cryptosporidium parvum	27	27.7	6.0
Norovirus G1	20	24.3	8.4
Norovirus G2	17	16.6	4.3
Astrovirus	30	30	—
Rotavirus	22	22	—
Sapovirus	32	32	—

[1] As detected by in-house PCR. [2] As detected by RidaGene PCR targeting EHEC/EPEC/*Shigella* spp./EIEC. [3] As detected by RidaGene PCR targeting ETEC and *Shigella* spp./EIEC. [4] As detected by PCR targeting the stable toxin. [5] As detected by PCR targeting the labile toxin.

2.3. Ethics. The surveillance described here was ordered as a preventive medical procedure by the German commanding hygiene officer of the EUTM Mali mission. Respective orders are legally covered by the self-administrative rights "Eigenvollzugskompetenz" of the German Armed Forces Medical Service regarding infectious disease prevention and control "Infektionsschutz". Collected data were assessed anonymously, thus avoiding any violation of §25 of the Declaration of Helsinki (DoH/Oct 2008) or national data protection laws "Bundesdatenschutzgesetz".

3. Results

3.1. Frequency of Diarrhea and Coverage. Based on the reports of the field doctors to the NATO Deployment Health Surveillance Capability (DHSC), an average weekly incidence of diarrhea among EUTM soldiers of 5.8 patients per week was calculated. The average number of deployed soldiers at risk was 480; the resulting average weekly incidence rate per 1,000 soldiers was 12.1. During the surveillance period of 37 weeks, a total of 53 stool samples from 51 EUTM soldiers with diarrhea were collected, resulting in an average of 1.4 cases per week. Accordingly, the coverage of the surveillance was about 24.1% of the registered diarrhea cases.

3.2. Diagnostic Results. Positive PCR results for diarrhea-associated pathogens could be detected in 43/53 patient samples. The five quantitatively dominating pathogens were EPEC ($n = 21$), ETEC ($n = 19$), EAEC ($n = 15$), Norovirus ($n = 10$), and *Shigella*/EIEC ($n = 6$), followed by *Cryptosporidium parvum* ($n = 3$), *Giardia duodenalis* ($n = 2$), *Salmonella* spp. ($n = 1$), Astrovirus ($n = 1$), Rotavirus ($n = 1$), and Sapovirus ($n = 1$). Of note, both detections of *Giardia duodenalis* were in the same patient, so copy-strain assessment occurred here. Median and mean cycle threshold (Ct) values as well as calculated standard deviations (SD) are given in Table 2. Of note, the lowest Ct values were detected for bacteria. Ct-values for *Shigella* spp./EIEC varied considerably depending on the primer-probe composition used, with lowest Ct-values in the RidaGene ETEC/EIEC kit and highest in the in-house approach.

DNA of two and more pathogens was detected in 23 of the samples, of three and more pathogens in 11 samples, of four and more pathogens in 2 samples, and of as many as five pathogens in 1 sample. Measured Ct values for the respective cases are given in Table 3. In several cases, low Ct-values are measured for more than one pathogen.

3.3. Norovirus Genotyping. Sequence analysis and genotyping verified the detection of Norovirus GII in six out of seven initially PCR-positive cases. The procedure failed for the seventh Norovirus GII detection and for all three cases positive for Norovirus GI. At least two out of three Norovirus GI-positive cases were confirmed by a separate real-time RT-PCR [17, 18] (data not shown).

Genotyping of the six sequenced Norovirus G2 strains revealed GII.P7 in four instances. Three out of those four strains showed identical sequences, suggesting either nosocomial transmission or a common source of infection. Epidemiological assessment showed that the respective samples were collected from three patients within a single week, making a mini-outbreak highly likely. In two out of six instances, Norovirus GII.P16 and GII.P4 var New Orleans were identified, respectively.

The underlying sequence information has been deposited and is freely accessible via http://www.rivm.nl/mpf/norovirus/typingtool/job/1197792283/.

3.4. Cultural Approach. From 13 out of the 48 analyzed stool samples, ESBL-positive Enterobacteriaceae were isolated by

Table 3: Ct-values in cases of multiple pathogen detections (in brackets). If both stable and labile toxin of STEC were detected, two Ct-values are given, otherwise only one. In case of *Shigella* spp./EIEC detections, three Ct-values are shown, reflecting the three applied PCR approaches. In several cases, low Ct-values are measured for more than one pathogen.

Case	Pathogen 1	Pathogen 2	Pathogen 3	Pathogen 4	Pathogen 5
1	*Shigella* spp./EIEC (Ct 13, 14, 16)	EPEC (Ct 18)	ETEC (Ct 13, 16)	EAEC (Ct 19)	Norovirus G1 (Ct 20)
2	*Salmonella* spp. (Ct 27)	EPEC (Ct 15)	Norovirus G2 (Ct 11)	Astrovirus (Ct 30)	—
3	EPEC (Ct 29)	EAEC (Ct 19)	Norovirus G2 (Ct 17)	—	—
4	ETEC (Ct 17, 20)	EAEC (Ct 19)	Norovirus G2 (Ct 14)	—	—
5	ETEC (Ct 13, 20)	EAEC (Ct 18)	Norovirus G2 (Ct 21)	—	—
6	*Shigella* spp./EIEC (Ct 12, 13, 14)	EPEC (Ct 21)	ETEC (Ct 21)	—	—
7	EPEC (Ct 17)	ETEC (Ct 20)	EAEC (Ct 19)	—	—
8	EPEC (Ct 20)	ETEC (Ct 13)	EAEC (Ct 21)	—	—
9	EPEC (Ct 16)	ETEC (Ct 13)	EAEC (Ct 22)	—	—
10	ETEC (Ct 15)	EAEC (Ct 15)	*Cryptosporidium parvum* (Ct 34)	—	—
11	EPEC (Ct 23)	ETEC (Ct 16, 21)	EAEC (Ct 22)	—	—
12	*Shigella* spp./EIEC (Ct 18, 20, 21)	EPEC (Ct 26)	—	—	—
13	EPEC (Ct 28)	*Giardia duodenalis* (Ct 28)	—	—	—
14	EPEC (Ct 21)	ETEC (Ct 17)	—	—	—
15	EPEC (Ct 25)	ETEC (Ct 12)	—	—	—
16	EPEC (Ct 22)	Norovirus G2 (Ct 17)	—	—	—
17	ETEC (Ct 11, 18)	EAEC (Ct 14)	—	—	—
18	ETEC (Ct 15)	EAEC (Ct 23)	—	—	—
19	EAEC (Ct 18)	*Cryptosporidium parvum* (Ct 22)	—	—	—
20	EPEC (Ct 22)	Sapovirus (Ct 32)	—	—	—
21	*Shigella* spp./EIEC (Ct 12, 23, 18)	EPEC (Ct 19)	—	—	—
22	EPEC (Ct 22)	ETEC (Ct 19, 19)	—	—	—
23	EPEC (Ct 21)	ETEC (Ct 24, 29)	—	—	—

thioglycolate broth enrichment with subsequent growth on ESBL selective agar. From 12 samples, ESBL-positive *Escherichia coli* were isolated with proof of more than one strain in two instances. The total number of ESBL-positive *E. coli* strains was 15. One of those strains was identified as EAEC by RidaGene PCR. An ESBL-positive *Klebsiella pneumoniae* strain was isolated from another sample.

For the 13 samples containing ESBL-positive Enterobacteriaceae, sensitivity against nonpenicillin, noncephalosporin antibiotics was determined by VITEK-II- and E-test-based resistance testing. If several ESBL-positive *E. coli* were simultaneously isolated, the most resistant strain with the resulting highest risk of selection under antibiotic pressure was chosen. Sensitivity for carbapenems, tigecycline, and fosfomycin was shown in all 13 cases; in 12 for nitrofurantoin, which is only suitable for urinary tract infections; in 11 for fluoroquinolones; and in 10 for gentamicin. Resistance against trimethoprim/sulfamethoxazole, which is frequently encountered in tropical settings, was demonstrated in all 13 cases.

3.5. Rep-PCR of ESBL-Positive Enterobacteriaceae. In total, 15 *E. coli* strains from 12 patients were subjected to rep-PCR-based DiversiLab typing. Within the 95% cutoff range for clonal identity, three clonal clusters comprising 7 strains from 6 patients were observed (Figure 1). This suggests the presence of common sources of infection or nosocomial

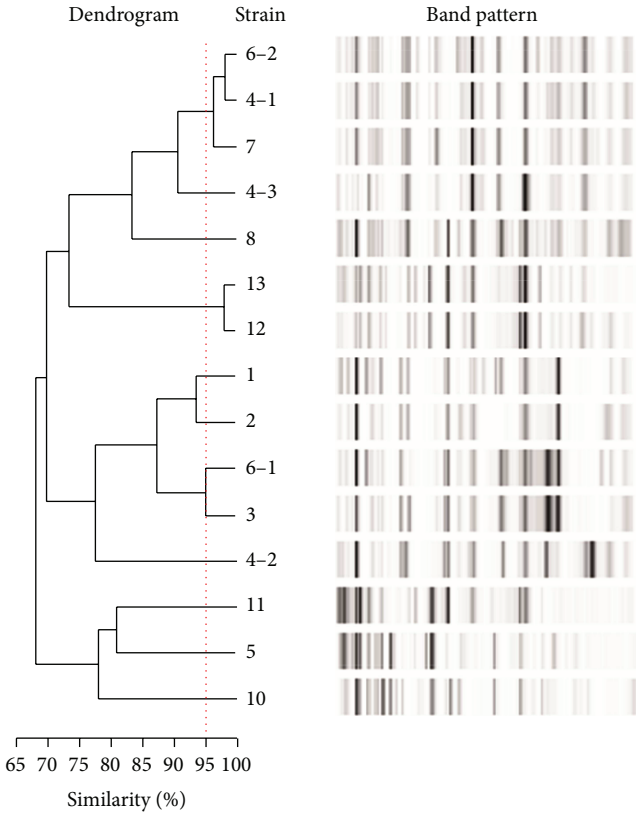

Figure 1: Dendrogram of the DiversiLab typing results of 15 ESBL-positive *E. coli* strains from 12 patients. The strains' labeling consists of the patient number (number before the "–") and the strain number in case of more than one isolated ESBL-positive *E. coli* strain per patient (number following the "–"). The strain of patient 9 is missing, as the respective ESBL-positive isolate was not *E. coli* but *K. pneumoniae*. Three clusters of clonal identity beyond the 95%-similarity cutoff (dotted red line) are detectable.

transmission within the camp. One patient was even colonized by *E. coli* strains from two different clusters (Figure 1). For the remaining 8 strains from 6 patients, clonal identity was excluded.

3.6. Clinical and Epidemiological Assessment. Clinical data were provided for 49 patients. The completeness of clinical data varied: missing data are characterized as "no data" in the tables in the following. In those 49 diarrhea patients, bacterial pathogens were detected in 34 instances, viral pathogens in 12 instances, and parasitic pathogens in 3 instances. No enteric pathogen was detectable in 9 out of these 49 patients with the applied procedures. DNA of more than one enteric pathogen was detectable in 23 of the soldiers with acute diarrhea.

3.6.1. Age, Gender, Nationality, Sites of Deployment, and Previous Stays in High-Endemicity Regions for Diarrhea-Associated Pathogens. No particular distribution pattern of bacterial, viral, and parasitic enteric pathogens was observed with respect to gender and nationality of the soldiers with diarrhea. Soldiers who were younger than 30 years of age showed coinfections with multiple enteric pathogens less frequently than older soldiers. Of note, all assessed diarrhea patients who were deployed to Bapho were infected by viral pathogens. Previous stays in high-endemicity settings did not have any notable effects on the acquisition of bacterial, viral, and parasitic pathogens (Table 4).

3.6.2. Mode of Food Intake. As few as 2 out of 15 diarrhea patients who claimed to have eaten exclusively in the field kitchen were free of enteric pathogens in the PCR analyses. Bacterial pathogens dominated in this group. Soldiers who restricted their diet to hotel food were prone to both bacterial and viral enteric infections. There was not a single diarrhea patient in this assessment who had eaten field rations alone prior to the onset of diarrhea (Table 5).

3.6.3. Stool Frequency and Consistency. A small proportion of 11 patients showed less severe symptoms with fewer than three unformed stools per day. Of note, no enteric pathogens were detected in only one of these patients. There was no obvious distribution pattern of bacterial, viral, or parasitic pathogens in these less severe diarrhea cases.

The observed stool consistency of collected samples, as reported by the laboratory technician, was considerably less unformed than the reported stool consistency. A total of 13 out of 49 stool samples were already hard at the time of sample collection, suggesting that the symptoms had already improved. In contrast, no patient reported formed stools to the field doctor. Only 3 out of these 13 formed stools were without detectable pathogen DNA at the time of assessment (Table 6).

3.6.4. Accompanying Symptoms including Fever. Light to moderate symptoms including nausea and vomiting, cramps, abdominal pain, and flatulence were frequent in the assessed diarrhea patients. Cramps, abdominal pain, and flatulence were particularly often detectable in diarrhea patients with bacterial infections, while nausea and vomiting were equally likely for both bacterial and viral infections. Only one instance of bloody diarrhea was observed in a patient with *Shigella* spp./EIEC as the only detectable pathogen in stool. Fever was confirmed in only three instances without any detectable association with a particular pathogen group (Table 7).

Of note, the distribution of accompanying symptoms did not change considerably if only the 34 patients who reported ≥3 stools per day were included into the assessment (Table 7).

Only one enteric pathogen per patient was detected in 16 patients who reported accompanying symptoms, comprising 4 cases with STEC, 2 cases with EAEC, 2 cases with EPEC, 2 cases with Norovirus G1, 2 cases with Norovirus G2, 2 cases with *Shigella* spp./EIEC, 1 case with *Cryptosporidium parvum*, and 1 case with Rotavirus, respectively. Bacterial infections were associated with a broad distribution of symptoms (Table 7). As expected, patients infected with enteroinvasive *Shigella* spp./EIEC showed a particularly wide spectrum of symptoms. Norovirus infections and *Cryptosporidium parvum* infections were associated with nausea, vomiting,

Table 4: Age, gender, nationality, sites of deployment, and previous stays in high-endemicity regions for diarrhea-associated pathogens of diarrhea patients with bacterial, viral, and parasitic infections.

	Detected pathogen groups				
	Bacteria	Viruses	Parasites	No detected pathogen	Multiple infections
Age					
<30 years	10/15	6/15	0/15	2/15	5/15
30–50 years	10/15	3/15	1/15	3/15	8/15
>50 years	2/2	1/2	0/2	0/2	2/2
No data	12/17	2/17	2/17	4/17	7/17
Gender					
Male	31/44	11/44	3/44	7/44	20/44
Female	3/5	1/5	0/5	2/5	2/5
Nationality					
Austria	1/1	1/1	0/1	0/1	1/1
Belgium	7/9	2/9	1/9	1/9	3/9
Colombia	1/1	0/1	0/1	0/1	0/1
Germany	17/25	8/25	0/25	4/25	12/25
Greece	3/3	0/3	1/3	0/3	3/3
Ireland	0/1	0/1	0/1	1/1	0/1
Italy	0/1	0/1	0/1	1/1	0/1
Portugal	0/1	0/1	0/1	1/1	0/1
Romania	0/1	1/1	0/1	0/1	0/1
Spain	4/4	0/4	0/4	0/4	2/4
Spain/Bolivia	0/1	0/1	0/1	1/1	0/1
No data	1/1	0/1	1/1	0/1	1/1
Site of deployment					
Koulikoro	31/41	7/41	3/41	6/41	19/41
Koulikoro and Bamako	1/2	1/2	0/2	1/2	1/2
Bamako	0/1	0/1	0/1	1/1	0/1
Bapho	2/4	4/4	0/4	0/4	2/4
No data	0/1	0/1	0/1	1/1	0/1
Previous stays in high endemicity settings					
Yes	8/13	3/13	1/13	2/13	4/13
No	26/36	9/36	2/36	7/36	18/36

and abdominal pain, and Norovirus G1 infections also were associated with cramps and flatulence (Table 7).

3.6.5. Antimalarial Prophylaxis, Hygiene Counseling, Vaccination against Cholera and Typhoid Fever, and Medication. Low-dose doxycycline-monohydrate antimalarial prophylaxis at 100 mg/day did not provide any protection against bacterial enteric infections. Among the diarrhea patients under doxycycline prophylaxis, 7 out of 10 were positive for DNA of bacterial enteric pathogens.

The vast majority of diarrhea patients were properly counseled regarding hygiene on tropical deployments and were vaccinated against typhoid fever. Infections with bacterial enteric pathogens were particularly frequent in patients who were vaccinated against cholera and typhoid fever. Use of anti-infective drugs was documented for three patients; all three took rifaximin and one in addition metronidazole. DNA of bacterial enteric pathogens was detectable in only one of these patients; no pathogen DNA was observed in two of them (Table 8).

4. Discussion

Risk assessment by standardized monitoring and surveillance of deployed soldiers in subtropical or tropical countries contributes to evaluation of both individual risk and preventive measures. As previously shown, infection risks with enteric pathogens increase if sophisticated hygiene precautions regarding food and drinking water cannot be maintained on military deployments, for example, in the case of small missions [3]. Considering an average clinical incidence of diarrhea between 5% and 7% per 100 per month

Table 5: Mode of food intake of diarrhea patients with bacterial, viral, and parasitic infections.

	Detected pathogen groups				
	Bacteria	Viruses	Parasites	No detected pathogen	Multiple infections
Field kitchen only	12/15	1/15	1/15	2/15	6/15
Field kitchen and field rations	1/1	0/1	0/1	0/1	1/1
Field kitchen and field rations and outdoor facility	2/4	0/4	1/4	1/4	2/4
Field kitchen and field rations and hotel and outdoor facility	2/2	0/2	0/2	0/2	0/2
Field kitchen and field rations and hotel and restaurant	1/1	1/1	0/1	0/1	1/1
Field kitchen and hotel	3/5	1/5	0/5	2/2	2/5
Field kitchen and hotel and outdoor facility	3/3	2/3	1/3	0/3	3/3
Field kitchen and outdoor facility	3/5	2/5	0/5	0/5	1/5
Field kitchen and restaurant	2/3	0/3	0/3	1/3	1/3
Hotel	4/7	5/7	0/7	1/7	4/7
Hotel and outdoor facility	0/1	0/1	0/1	1/1	0/1
Restaurant	1/1	0/1	0/1	0/1	1/1
No data	0/1	0/1	0/1	1/1	0/1

Table 6: Stool frequency and consistency in diarrhea patients with bacterial, viral, and parasitic infections.

	Detected pathogen groups				
	Bacteria	Viruses	Parasites	No detected pathogen	Multiple infections
Stool frequency					
1-2 stools per day	8/11	5/11	1/11	1/11	7/11
≥3 stools per day	23/34	6/34	2/34	7/34	14/34
No data	3/4	1/4	0/4	1/4	1/4
Stool consistency as described by the patient					
Watery	18/26	8/26	3/26	3/26	12/26
Mushy	4/5	0/5	0/5	1/5	2/5
Changing	2/3	0/3	0/3	1/3	2/3
Watery/mushy	4/5	1/5	0/5	1/5	2/5
Watery/changing	0/1	1/1	0/1	0/1	0/1
Slimy/changing	0/1	0/1	0/1	1/1	0/1
No data	6/8	2/8	0/8	2/8	4/8
Stool consistency as observed by the technical assistant					
Hard	10/13	1/13	1/13	3/13	6/13
Watery	11/14	2/14	1/14	1/14	7/14
Mushy	12/17	9/17	0/17	1/17	8/17
Slimy	1/2	0/2	1/2	1/2	1/2
No data	0/3	0/3	0/3	3/3	0/3

on military deployments [19], the estimated average weekly incidence rate of 12.1 per 1,000 soldiers for the EUTM forces is not surprising. The slightly lower incidence might be attributable to the comparably good hygiene standards in Camp Koulikoro.

In the surveillance of deployed European soldiers with diarrhea in tropical Mali described here, noninvasive EPEC, ETEC, and EAEC clearly predominated, followed by Norovirus and *Shigella* spp./EIEC, while other invasive bacteria and protozoa were less frequent. The surveillance

Table 7: Accompanying symptoms in diarrhea patients with bacterial, viral, and parasitic infections. n.o. = not observed.

	Detected pathogen groups				
	Bacteria	Viruses	Parasites	No detected pathogen	Multiple infections
Accompanying symptoms in all patients					
Nausea/vomiting	8/15	7/15	0/15	4/15	6/15
Cramps	15/20	6/20	2/20	2/20	10/20
Abdominal pain	19/29	8/29	2/29	5/29	10/29
Flatulence	9/13	4/13	1/13	2/13	6/13
Bloody diarrhea	1/1	0/1	0/1	0/1	0/1
No data	9/12	1/12	1/12	3/12	7/12
Fever					
Yes	2/3	1/3	0/3	1/3	2/3
No	32/46	11/46	3/46	8/46	20/46
Accompanying symptoms in patients with ≥ 3 stools per day (n = 34)					
Nausea/vomiting	4/9	2/9	0/9	4/9	2/9
Cramps	12/16	3/16	2/16	2/16	8/16
Abdominal pain	15/22	5/22	2/22	4/22	8/22
Flatulence	6/9	3/9	1/9	2/9	5/9
Bloody diarrhea	1/1	0/1	0/1	0/1	0/1
No data	5/7	0/7	0/7	2/7	3/7
Fever					
Yes	2/3	1/3	0/3	1/3	2/3
No	21/31	5/31	2/31	6/31	12/31
Accompanying symptoms in patients with only 1 detected pathogen and assessed symptom data (n = 16)					
Nausea/vomiting	EPEC (1/2), *Shigella* spp./EIEC (1/2)		Norovirus G2 (2/2), Norovirus G1 (1/2)	*Cryptosporidium parvum* (1/1)	
Cramps	*Shigella* spp./EIEC (2/2), STEC (3/4)		Norovirus G1 (2/2)	n.o.	
Abdominal pain	EAEC (2/2), EPEC (2/2), *Shigella* spp./EIEC (2/2), STEC (3/4)		Norovirus G1 (2/2), Norovirus G2 (2/2)	*Cryptosporidium parvum* (1/1)	
Flatulence	EAEC (1/2), *Shigella* spp./EIEC (1/2), STEC (1/4)		Rotavirus (1/1), Norovirus G1 (1/2)	n.o.	
Bloody diarrhea	*Shigella* spp./EIEC (1/2)		n.o.	n.o.	
Fever					
Fever was not observed.					

interval included periods of both dry season and rainy season, when diarrhea is usually more frequent. Multiplex real-time PCR proved to be a suitable platform for the identification of multiple pathogens in parallel assays, thus allowing for a rapid diagnosis with subsequent enforcement of adequate hygiene precautions. Of note, demonstration of pathogen DNA was still possible in the subacute state when the stool consistency had already changed from fluid or mushy to hard.

Diarrhea-associated *E. coli* strains are frequent in tropical settings as previously shown [20–23], so the dominance of EPEC, ETEC, and EAEC is not surprising. Recent studies further stress the importance of enteropathogenic viruses, in particular Norovirus, in tropical settings [21, 24]. Due to its high contagiousness and tenacity [25, 26], Norovirus is particularly prone to causing local outbreaks [27]. Accordingly, its rapid and reliable identification is of use in military

Table 8: Antimalarial prophylaxis, hygiene counseling, vaccination against cholera and typhoid fever, and medication of diarrhea patients with bacterial, viral, and parasitic infections.

	Detected pathogen groups				
	Bacteria	Viruses	Parasites	No detected pathogen	Multiple infections
Malaria prophylaxis					
Doxycycline	7/10	2/10	1/10	2/10	3/10
Atovaquone/proguanil	18/26	8/26	1/26	4/26	12/26
Mefloquine	8/9	2/9	1/9	0/9	6/9
Switch atovaquone/proguanil to doxycycline	1/1	0/1	0/1	0/1	1/1
None	0/1	0/1	0/1	1/1	0/1
No data	0/2	0/2	0/2	2/2	0/2
Vaccines					
Typhoid fever	23/32	7/32	3/32	5/32	15/32
Cholera and typhoid fever	7/10	1/10	0/10	3/10	4/10
No data	4/7	4/7	0/7	1/7	3/7
Medication					
Antibiotics	1/3	0/3	0/3	2/3	0/3
No antibiotics or uncertain	33/46	12/46	3/46	7/46	22/46
Hygiene counseling					
Yes	28/39	7/39	3/39	7/39	17/39
No	1/1	0/1	0/1	0/1	0/1
No data	5/9	5/9	0/9	2/9	5/9

deployments to allow for a rapid enforcement of appropriate hygiene precautions.

Norovirus genotyping confirmed the worldwide occurrence of different genotypes that lead to outbreaks under conditions of restricted hygiene. As shown for three patients with identical Norovirus sequences who became symptomatic within a single week, a single source of infection or person-to-person transmission due to low hygienic compliance may easily affect several soldiers on deployment. Moreover, genotype II.4 is a pandemic strain, which has a high potential to cause nosocomial outbreaks [28]. Thus the sequencing results obtained, confirming at least one small outbreak event, stress the importance of rapid Norovirus diagnostics on deployment.

The inability of genotyping in initially positive tested samples is a consequence of the high mutation rate in Norovirus. In contrast, depending on the targeted sequence, false positive results of Norovirus PCR tests may occur [29]. However, this was not confirmed in this study.

The relative lack of enteroinvasive bacteria and enteropathogenic protozoan parasites was consequently associated with predominantly mild to moderate symptoms in diseased soldiers. Only one case of bloody diarrhea in a patient with *Shigella* spp./EIEC as the only detectable pathogen in the stool sample was observed. *Salmonella* spp. was detected in one instance only; *Campylobacter jejuni* was not observed at all. This speaks in favor of the locally practiced food and drinking water hygiene, as enteroinvasive bacteria [30, 31] and enteropathogenic protozoa [32–35] are frequent causes of severe gastrointestinal infections in sub-Saharan Africa.

The frequent occurrence of bacterial diarrhea in soldiers who ate exclusively at the field kitchen suggests autochthonous spread of pathogens [36, 37] within the field camp, for example, by smear infection. A possible reason could be inadequate toilet hygiene. Also, occasional hygiene problems in the field kitchen could not be excluded, because no soldier with diarrhea reported exclusive consumption of standardized field rations, for which the risk of acquiring gastrointestinal infections is virtually zero.

The data regarding the food sources of the infected soldiers should be interpreted with care. As uncontrolled food consumption outside military infrastructure might pose a disciplinary offense, interpretability of respective information on the questionnaire is limited by a reporting bias. Accordingly, it cannot be excluded that a considerable proportion of diarrhea patients who claimed to have exclusively eaten at the field kitchen indeed consumed food from outside the camp as well.

In spite of a reported partial protective effect of cholera vaccination against travelers' diarrhea [38], detection of DNA of enteropathogenic bacteria was particularly frequent in the cholera-vaccinated soldiers.

As a further result, the surveillance impressively demonstrates the potential multicausal etiology of acute diarrhea on tropical deployments, which has to be considered if targeted therapy of a specific identified pathogen fails. Asymptomatic

pathogen carriage was not excluded but is unlikely because the deployed soldiers analyzed did not arrive from high-endemicity settings. As is typical for surveillance analyses, no stool samples were collected prior to deployment, which would have allowed for comparison testing; this is an undeniable limitation of the data presented.

The inclusion of patients into the surveillance merely based on the subjective assessment of the local field doctor is a major limitation of the study. This limitation does not allow direct comparisons with studies using standardized definitions of travelers' diarrhea, for example, including stool quality assessments like the Bristol stool scale [39–42]. Of note, the assessment of symptoms of patients with ≥3 stools per day led to similar results as observed for the whole study population. The focus of the surveillance was on patients with gastrointestinal symptoms leading to incapacitation from military duty, not on patients meeting a standard definition of travelers' diarrhea. Therefore, such a nonconventional inclusion strategy was chosen.

PCR is a highly sensitive method for the detection of enteric pathogens in stool, outperforming alternative approaches such as microscopy regarding the detection limit [5, 43]. However, the problem of simultaneously detecting several enteric pathogens by PCR in stool samples in high-endemicity settings for diarrheal disease is a constant stumbling block, because it hinders etiological attribution and subsequent targeted antimicrobial therapy in case of severe disease. Here we could demonstrate that this problem also applies to European soldiers deployed in the tropics.

Quantitative PCR tests have been suggested as useful tools for a more reliable attribution of etiological significance to detected enteric pathogens [44, 45], discriminating active infection from asymptomatic carrier status or shedding of residual pathogen DNA after previous, already cleared infections. However, no generally accepted standards for such quantitative approaches have been established so far. DNA quantification in complex materials like stool samples is further limited by various degrees of PCR inhibition [46]. In this surveillance, low Ct-values, potentially suggesting etiological relevance, were observed for more than one pathogen in several instances. Sufficiently powered future studies will be necessary to evaluate the usefulness of quantitative stool PCR and the definition of reliable cut-off values for the diagnostic routine.

However, etiological attribution is not the only aspect that makes calculated antimicrobial therapy challenging in case of severe diarrhea in soldiers on deployment. Knowledge about the local antimicrobial-resistance situation in diarrhea-associated bacteria is crucial to allow for a tailored antimicrobial therapy. Next to standard recommendations regarding the therapy of acute gastroenteritis [47] and traveler's diarrhea [48–52], the British and U.S. military medical services also intend studies on the optimization of single-dose antibiotic treatment regimens [19].

High rates of colonization with atypically resistant or even multidrug-resistant bacteria in returnees from tropical settings have recently been described [53–55]. Increased colonization with multidrug-resistant bacteria in the tropics can be triggered by prescribing antibiotics for travelers' diarrhea [56, 57]. However, during the International Security Assistance Force (ISAF) mission in Afghanistan, colonization of German soldiers with ESBL-positive Enterobacteriaceae was as low as 5% [58], despite considerably higher colonization rates in Afghan patients. In contrast, nearly every fourth stool sample of European soldiers demonstrated ESBL colonization in our present surveillance during the EUTM Mali deployment. Although rep-PCR suggests a moderate degree of clonal diversity of ESBL-positive strains from Mali, several clonal mini-clusters suggest either fecal contamination of common sources of infection or nosocomial spreading within the field camp.

In persons of weakened immunological state (e.g., after polytrauma on deployment), transition of enteric bacteria through the gut tissue with resulting sepsis may occur. If resistant bacteria enter blood circulation in this way [59–61], antibiotic therapy becomes challenging. The probability of such events rises in case of high colonization rates with resistant bacteria and selective pressure due to antibiotic therapy or prophylaxis. It is a well-documented phenomenon that colonizing resistant bacteria can cause blood stream infections under the selective pressure of antibiotics [62–66].

The high incidence of ESBL-positive Enterobacteriaceae in deployed soldiers in Mali suggests the use of alternative antibiotic drugs in case of systemic infections. According to German recommendations, oxyimino-cephalosporins (e.g., ceftazidime) or aminoacyl penicillin-beta-lactamase combinations (e.g., piperacillin/tazobactam) are appropriate substances for calculated initial therapy of sepsis [67]. However, these substances will fail in case of sepsis due to ESBL-positive Enterobacteriaceae. Furthermore, resistance against the orally administrable fluoroquinolones was observed in several instances, making the use of intravenous reserve substances such as carbapenems unavoidable if severe systemic bacterial infections occur.

Of note, increasing antibiotic resistance has recently been described for diarrhea-associated *E. coli* and *Shigella* spp. as well [68]. In this study, only one ESBL-positive EAEC was isolated.

No efficient procedures for reliable eradication of enteric colonization with ESBL-positive Enterobacteriaceae have been described so far. Accordingly, a high probability of such colonization in returnees from Mali has to be considered both for hygienic reasons and for the choice of antibiotic drugs in case of future systemic infections.

5. Conclusions

Real-time multiplex-PCR systems proved to be useful for diarrhea surveillance in the tropical deployment setting, allowing the detection of enteric pathogens in more than 80% of the analyzed stool samples of European soldiers in Mali. However, the frequent detection of DNA of several pathogens in high-endemicity settings impedes the etiological attribution. Noninvasive enteropathogenic bacteria and Norovirus dominated quantitatively and were associated with mild to moderate symptoms. The reported mode of food intake suggests the presence of transmission routes in the field camp.

Conflict of Interests

The authors declare that there is no conflict of interests according to the guidelines of the International Committee of Medical Journal Editors.

Authors' Contribution

Hagen Frickmann and Philipp Warnke contributed equally to this work.

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Research Article

Sampling and Homogenization Strategies Significantly Influence the Detection of Foodborne Pathogens in Meat

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Efficient preparation of food samples, comprising sampling and homogenization, for microbiological testing is an essential, yet largely neglected, component of foodstuff control. *Salmonella enterica* spiked chicken breasts were used as a surface contamination model whereas salami and meat paste acted as models of inner-matrix contamination. A systematic comparison of different homogenization approaches, namely, stomaching, sonication, and milling by FastPrep-24 or SpeedMill, revealed that for surface contamination a broad range of sample pretreatment steps is applicable and loss of culturability due to the homogenization procedure is marginal. In contrast, for inner-matrix contamination long treatments up to 8 min are required and only FastPrep-24 as a large-volume milling device produced consistently good recovery rates. In addition, sampling of different regions of the spiked sausages showed that pathogens are not necessarily homogenously distributed throughout the entire matrix. Instead, in meat paste the core region contained considerably more pathogens compared to the rim, whereas in the salamis the distribution was more even with an increased concentration within the intermediate region of the sausages. Our results indicate that sampling and homogenization as integral parts of food microbiology and monitoring deserve more attention to further improve food safety.

1. Introduction

Despite the rise of novel molecular and high-throughput detection methods, the recovery, isolation, and enumeration of bacterial pathogens in food are still primarily based on culture techniques, the current gold standard in food microbiology [1, 2]. The continuing dominance of traditional microbiological detection methods in foodstuff control is attributed to the goal to prove the absence or presence of living pathogenic bacteria, which is indispensable to assess the actual health hazard for consumers. The mere presence of bacterial DNA, which represents the target for many rapid techniques like PCR, cannot predict the risk of infection. However, the formation of visible colonies requires the successful recovery of the target bacteria out of a food matrix in a viable and replication-competent state. Thus, sample preparation is critical for the successful subsequent microbiological detection and has to be adapted to the respective food matrix [3, 4].

The initial extraction of the pathogen is usually performed by applying mechanical forces of varying magnitude to homogenize the food matrix [5, 6]. In addition to simple procedures such as vortexing or manual release, various technical solutions are commercially available. Peristaltic blenders like the Stomacher or, alternatively, the Pulsifier are probably the most prominent ones for microbiological detection [7–9]. The widely used Stomacher consists of two quickly movable paddles, which disperse the input food sample/buffer mix for enrichment via cultivation. Other homogenization methods, using beads to mill food or the application of ultrasound, might be also employed for this purpose. Such applications may be also used for the disruption of cells to release proteins or DNA for molecular detection techniques or further downstream purification

processes [10, 11]. Many novel systems (e.g., FastPrep-24) also offer the possibility of adapting the homogenization device to the food matrix by adding further components like quartz sand or beads of variable sizes. The effects of these diverging approaches of sample pretreatment on cell viability and test sensitivity have been insufficiently investigated so far. Furthermore, the instructions in the highly standardized and widely accepted ISO standards for the identification of microbes in food are usually rather vague and unspecific with respect to the sampling process as well as the sample pretreatment and homogenization (in contrast to the downstream detection procedures). Thus the current use mainly depends on the availability of the devices mentioned above as well as on personal preferences and rarely considers the physical properties of the food matrix. Due to limited resources, some laboratories may entirely rely on manual homogenization or simple vortexing.

Bacterial pathogens, such as *Salmonella enterica*, might be located on the surface of a food product due to cross contamination during slaughtering in case of meat or during harvest and subsequent transport. In contrast, processed products like sausages or cheese can get contaminated inside the food product during the production process [12–14]. Little is known about the nature of the microbial burden, whether it is evenly distributed throughout the entire product or whether a microbial gradient towards the surface is present. In the latter case, an arbitrarily taken sample might cause false-negative results. Likewise, the use of different homogenization approaches for the extraction of inner microbial contamination and the microbial survival rate after the imposed shear forces have not been systematically compared and neither has the physical detachment of bacteria from the surface been evaluated [15, 16]. In case of *Salmonella* contaminated fresh produce as well as pathogenic bacteria on fish, studies indicate the significance of sample preparation [17–19].

In this study, the applicability of four different mechanical homogenizing devices (stomaching by Bagmixer 400, milling by FastPrep-24 or SpeedMill, and sonication by the Branson Sonifier, Table 1) for pathogen isolation and conventional detection by cultivation for processed and unprocessed meat products was evaluated. As a proof of principle, using *Salmonella enterica*, surface contamination was established on chicken breasts and inner-matrix contamination was established in pork sausages of soft and hard consistence. The microbial survival rate of this Gram-negative pathogen and the recovery success of each method were assessed. Additionally, the influence of sample taking on the test outcome was investigated.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. Exemplarily for Gram-negative Enterobacteriaceae, the *Salmonella enterica* serovar Typhimurium (*S. enterica*) reference strain DSM 11320 (DSMZ, Braunschweig, Germany) was used for all experiments. *S. enterica* was cultivated under aerobic conditions at 37°C in lysogeny broth (LB) medium. For spiking experiments, aliquots of overnight cultures were transferred into fresh LB and cultivated under rotational shaking (GFL shaking incubator 3033, 180–200 rpm) until an optical density (OD_{588}) of 0.5 was reached. These cultures were serially diluted using 1% buffered peptone water (w/vol). Bacterial titers were enumerated after plating 100 μL of the dilution steps on selective media (Xylose Lysine Deoxycholate (XLD) agar, Oxoid, Wesel, Germany) and incubation for 22–26 h at 37°C.

2.2. Spiking of Food Products. Two types of meat contamination were simulated: surface contamination and inner-matrix contamination in which the pathogen can be distributed throughout the entire food matrix. In all food samples the absence of *Salmonella* prior to spiking was confirmed according to DIN EN ISO 6579:2002 (microbiology of food and animal feeding stuffs-horizontal method for the detection of *Salmonella* spp.). Only sporadic occurrence of other bacteria such as *Serratia*, *Hafnia*, or *Citrobacter* was found.

For artificial surface contamination, chicken breasts purchased at local supermarkets (Berlin, Germany) in January 2014 were used. One or two cubical-shaped pieces of chicken breast were cut using sterile equipment and razors to obtain meat samples with a weight of 4 g (BagMixer 400, Interscience, Germany), 3 g (FastPrep-24 (MP Biomedicals, France) and sonication), and 0.15 g (SpeedMill, Analytik Jena AG, Germany). On the surface of the samples, peptone water containing *Salmonella* (volume addition of 360 μL for stomaching, 270 μL for FastPrep-24 and sonication, and 135 μL for SpeedMill) was evenly applied to obtain spiked samples with a final bacterial load of approximately 3×10^5 CFU/g, corresponding to 3×10^4 CFU/mL in the homogenate. These samples were then incubated for 1 h at 4°C. Meat samples without artificial contamination were used as negative controls.

For the simulation of inner-matrix contamination, coarsely ground, smoked, or air-dried salami with a high degree of hardness and German Mettwurst (meat paste), a finely ground spreadable sausage of soft consistency, was produced in the technology facilities of the Federal Institute for Risk Assessment. These sausages, made of lean pork and bacon, were spiked under BSL-2 conditions within the production process with *Salmonella* in six different concentrations ranging from 1 CFU/g to 10^8 CFU/g. One preparation without *Salmonella* was used as negative control. Briefly, 3.7 kg of meat (2.4 kg lean pork, 1.3 kg bacon) was mixed with 50 mL of *Salmonella* solution containing the respective pathogen concentration while being minced and flavoured (90 g nitrite salting mix, 12 g paprika, and 8 g black pepper) in an automated meat cutter (HFM Fleischereimaschinen, Germany). Smoked salamis were cured for five days at 18°C in the Bastra MC 500; German Mettwurst was cured for 2 h and afterwards allowed to ripen for six days at 18°C, before being stored at −20°C until use.

2.3. Homogenization Procedure and Sausage Sampling. To evaluate the efficacy of various methods to homogenize meat and meat products, four devices with diverging technical approaches were systematically compared (Table 1).

Table 1: Properties of the chosen homogenization devices.

Method	Stomaching (Bagmixer 400)	FastPrep-24	SpeedMill	Branson Sonifier 450
Principle	Blending by movable paddles	Bead-mediated milling	Bead-mediated milling	Sonication
Handling	+	+	+	+/−
Portability and on-site usage	−	−	+	−
Adaptability to different matrices	+/−	++*	++*	+/−
Current usage for detection by cultivation	++	−	−	−
Parallel sample preparation	−	+ (2–48)**	+ (2–20)	−
Suitability for high volumes***	++ (<400 mL)	+ (<50 mL)	− (<2 mL)	+ (<50 mL)
Available volume range***	+/−	+	+/−	+
Avoidance of heat generation	+	+/−	+/−	+/−
Performance in this study				
Surface contamination	+	+	+	+
Inner-matrix contamination	Variable	+	+/−	−

++: excellent, +: good, +/−: ambiguous, and −: poor.
*Various matrix-specific kits and beads for sample preparation are commercially available.
**The parallel preparation of 48 samples is only possible for volumes smaller than 2 mL. Two samples can be homogenized simultaneously for the highest volume input.
***Exact volumes depend on the sizes of the used bags, BD Falcon tubes, and lysis tubes.

The SpeedMill is a small portable milling apparatus, suitable for on-site sample processing and easily adaptable to different sample types. Likewise, FastPrep-24 is a large-volume milling tool (adaptable also to small-volume samples), which also offers a broad range of modular adaptations by adding quartz sand or beads. Stomaching was chosen because it is one of the most frequently used ways to prepare food samples. Stomaching is considered as a gentle homogenization method since heat development is marginal and the peristaltic movements of the paddles distribute the input energy on a large area, reducing potential peaks in shear forces. Finally, sonication is a rather old, but simple, method, which exerts a distinct kind of mechanical force compared to stomaching or milling.

All food samples were diluted 1 : 10 in buffered peptone water (thus 36 mL for stomaching; 27 mL for sonication and FastPrep-24; 1.35 mL for homogenization in the SpeedMill). For stomaching, samples were placed in sterile stomacher bags (BagPage 400 mL, Interscience). Homogenization was performed at the highest intensity (paddle distance 7 mm) at indicated time intervals. Milling was performed with the FastPrep-24 system at a velocity of 5 m/s (500 Watts) and with the SpeedMill (150 Watts). In case of the FastPrep-24 device, BD Falcon 50 mL tubes (BD Biosciences, Germany) were filled with three ceramic beads with a size of 6.35 mm (1/4″ Ceramic Sphere MP). For the SpeedMill, 2 mL innuSpeed Lysis tubes E with 2.4–2.8 mm ceramic spheres (Analytik Jena) were employed. Sonication was performed under continuous cooling with circulating chilled water at the highest intensity (output power 400 Watts) in BD Falcon 50 mL tubes placed in the water-filled cup horn of a Branson Sonifier 450 (Branson Ultrasonics). An initial aliquot was taken before homogenization after vortexing for 20 s. Further aliquots were taken after 30 s and 1, 2, 4, and 8 min of homogenization. As the FastPrep-24 system requires a cooldown period of 5 min after 1 min of homogenization, food samples were cooled on ice for this time period after each minute of homogenization. All aliquots were diluted appropriately in buffered peptone water and plated twice in suitable dilution steps on selective medium. After an incubation period of 24 h at 37°C, colonies on XLD were enumerated. To monitor the accompanying flora of the food products, 100 μL of samples was also plated on LB agar and cultivated under aerobic and microaerobic conditions. Ambiguous colony morphologies and sporadically observed putatively accompanying flora on XLD agar plates were analyzed by the MALDI Biotyper (Bruker, Germany), following the instructions of the manufacturer. All homogenization experiments were performed in three independent tests.

To compare the different homogenization devices for the inner-matrix contamination, whole cross sections of the sausages were used, covering all regions of the sausages. To determine the spatial distribution of *Salmonella* within the sausages, four different regions were investigated, the inner core (A), the outer rim (B), whole cross sections covering the entire area of the sausage (C), and the intermediate region (D) without outer rim and inner core. For the inner core, sausage slices were cut and circular center pieces with a diameter of approximately 7 mm were taken. For the rim, the outer 3 mm of the sausages was used. A total of 3 g out of each region was diluted 1 : 10 in buffered peptone water and homogenized via 8 min of FastPrep-24 treatment as described above. All distribution experiments were again performed in three independent tests.

2.4. Statistical Analyses. Two-tailed unpaired Student's t-test was used to evaluate the significance of the results, assuming unequal variance between the two compared groups; P values below 0.05 were considered as significant. All data are given as means with standard deviations.

3. Results and Discussion

3.1. Surface Contamination. To compare and evaluate the four different homogenization approaches (Table 1), we first determined the release of bacteria from chicken breast surfaces artificially spiked with *S. enterica*. Immediately after 20 s of vortexing, considerable numbers of *Salmonella* were already found in the medium without homogenization. However, 8 min of homogenization resulted in an increase of the number of bacteria released after stomaching and FastPrep-24 treatment, whereas sonication and SpeedMill treatment led to a decrease in pathogen recovery compared to the CFU counts obtained after an initial rinse and vortexing step (Figure 1). Independent of the applied method, there was no significant difference between the recovery rates of bacteria from homogenized and nonhomogenized samples ($P > 0.05$). However, the intermethod comparison revealed a rather good performance of both FastPrep-24 and stomaching, on the one hand, and CFU losses by sonication and SpeedMill, on the other hand. Notably, the different methods differed substantially in the degree of sample disintegration as well as in the amount of generated foam, which hampers accurate pipetting. SpeedMill, and to a lesser extent FastPrep-24, produced the greatest amounts of debris and foam, while sonication causes only a slow increase in liquid turbidity and only limited fragmentation of the food samples.

To rule out that the homogenization procedure by itself exhibits a negative effect on the viability of *S. enterica*, thereby explaining the inferior results of SpeedMill and sonication, we investigated the effects on pure *Salmonella* cultures, diluted in buffered peptone water. No CFU loss was found for SpeedMill, even after 8 min of homogenization; in contrast, sonication of pure cultures for 8 min diminished the CFU count by roughly one-fourth, in accordance with the reduction seen for the surface contamination of chicken breast. This might indicate that the inferior performance of sonication is indeed a result of slow bacterial killing while SpeedMill treatment does not affect bacterial viability in pure cultures but seems to be unable to separate bacteria efficiently from the food surface. The results obtained for *S. enterica* are probably valid for other disease-causing members of the Enterobacteriaceae like *Escherichia coli* or *Yersinia* spp. In addition, it is likely that smaller bacteria like *Campylobacter* or Gram-positive pathogens like *Listeria monocytogenes* are also not affected in their viability after extensive homogenization because mechanical shear forces in general are more harmful for larger objects than for smaller ones. However, smaller beads and sphere materials other than ceramic might exhibit much more unfavorable shear forces. In accordance with this assumption, the manufacturers of FastPrep-24 and SpeedMill, MP Biomedicals and Analytik Jena, recommend the use of considerably smaller beads to lyse bacteria for subsequent molecular detection methods.

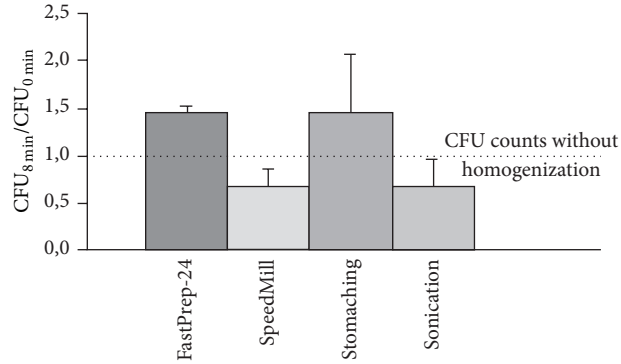

Figure 1: Changes in pathogen detection of chicken surface contamination after homogenization. The indicated bars express the normalized pathogen concentrations released from spiked chicken breast samples after 8 min of homogenization in relation to the CFU count after 20 s of sole vortexing.

In contrast to inner-matrix contamination, for which only scarce information is available so far, it has been reported that, for surface contamination, extended periods of stomaching and other homogenization methods did not significantly enhance the detection of the pathogen compared to simple rinsing procedures [11, 15, 20]. It was suggested by Sharpe and others that this might be the result of a "mass action" effect which prevents a higher pathogen release after equilibrium between the liquid phase and the food surface has been reached. Although this hypothesis has not yet been proven, it illustrates that longer homogenization periods and harsh homogenization methods are not necessary for surface contamination; instead soaking, hand-massaging, or the use of swabs for those food products might be equally applicable or even better procedures [6, 18, 21, 22].

3.2. Inner-Matrix Contamination. To elucidate the efficiency of mechanical disruption for the detection of inner-matrix contamination, two sausages with an artificial *Salmonella* contamination were produced. During the production, ripening and storage of the spiked sausages, the number of pathogens in the sample dropped by 3 to 4 log units. In the salamis, slightly higher concentrations of *S. enterica* compared to the meat paste were found despite being spiked in equal amounts, which might be a result of the shorter curing and ripening period (in total, five days for the salamis and six days for the meat paste). For the homogenization experiments, sausages primarily spiked with 10^8 CFU/g were chosen to yield sufficient CFU counts for an adequate interpretation. In contrast to surface contamination, the two sausage types showed substantial differences in terms of pathogen release (Figure 2). For the soft, finely minced meat paste, FastPrep-24, the large-volume milling device, showed a superior performance, extracting seven times more pathogens after 8 min than stomaching (Figure 2; left column). The low-volume SpeedMill enabled intermediate extraction success, whereas sonication, which was unable to substantially break up the sausage matrix, yielded no CFUs at all (data not shown). Interestingly, independent

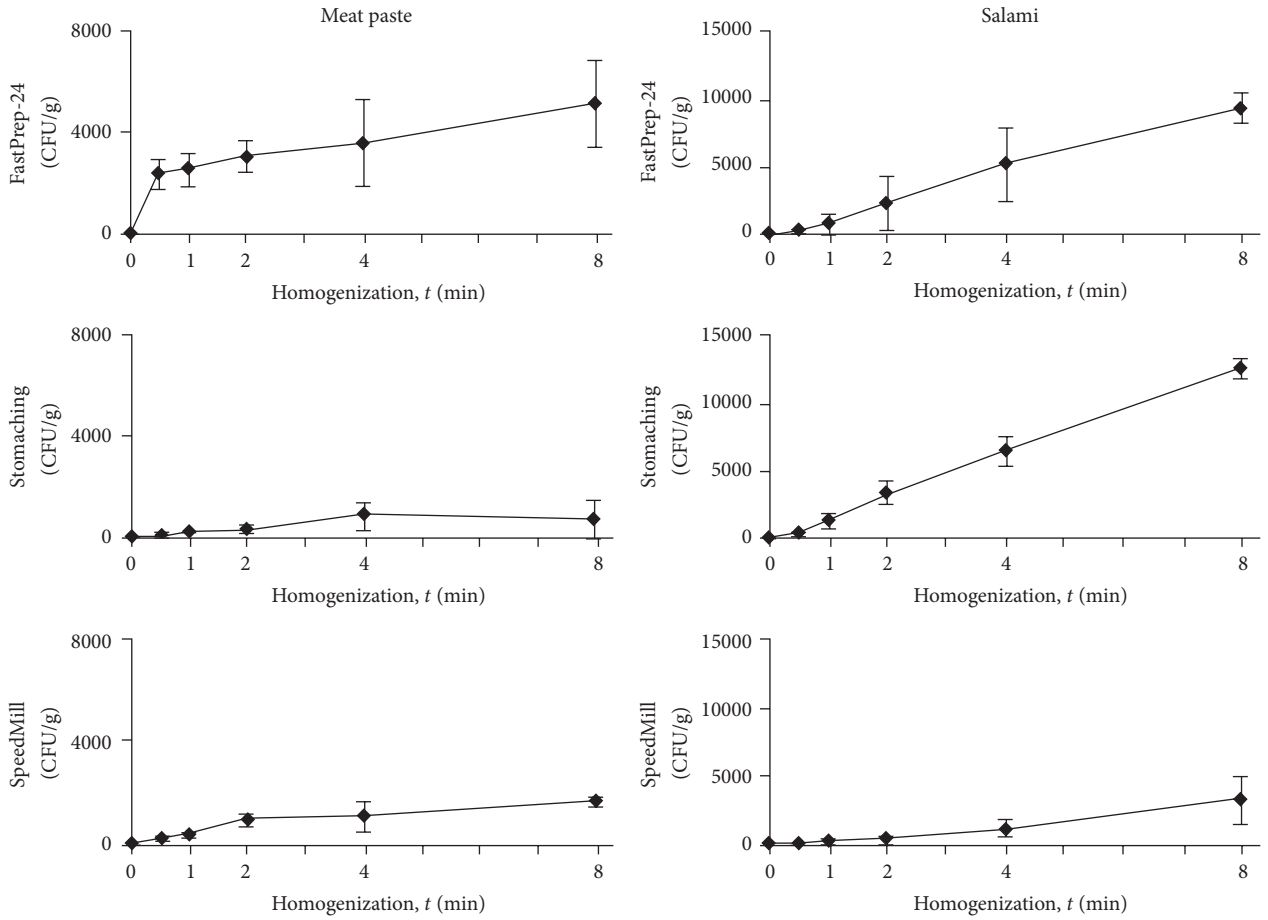

Figure 2: Homogenization of inner-matrix contamination. Release of *Salmonella* from whole cross sections of internally contaminated sausages after pretreatment by FastPrep-24, stomaching, and SpeedMill for 0, 30 s and 1, 2, 4, and 8 min was monitored.

of the system, the longer the homogenization period was chosen, the higher the number of detectable CFUs was, although a burst of pathogen release was already found after 30 s of homogenization. Simple vortexing and rinsing procedures (before homogenization; $t = 0$) yielded no CFUs, demonstrating that this procedure is only suitable for surface contamination. For the hard, coarsely ground salami, FastPrep-24 treatment and stomaching showed a comparable release of *S. enterica* (Figure 2; right column). In contrast to the meat paste, no initial burst of pathogen release was detected. Instead, after a short lag phase a continuous, nearly linear release rate of bacteria was measured. After shorter homogenization periods, remarkably less bacteria were extracted than after longer treatment; for example, the number of extracted bacteria was 10-fold less after 1 min compared to 8 min. Similar to the meat paste, SpeedMill treatment of the salamis was rather ineffective and sonication was not able to homogenize the matrix effectively. In both sausages the amount of accompanying flora was marginal, indicating that the influence of other bacteria did not play a major role. A third sausage type, smoked salami, showed results similar to the air-dried salami. However, due to the very low *Salmonella* concentration after the smoking process (data not shown), a statistically reliable comparison was not possible.

For routine examinations of food products, homogenization is usually performed for a rather short duration (e.g., 1-2 min) to save time and by using soft mechanical treatment (stomaching or blending at low intensities) to avoid loss of bacterial viability. However, the conventional mild sample preparation is not necessarily preferable because the results in Figure 2 show that longer treatments and harsher conditions are beneficial to determine inner-matrix contamination and do not affect the bacteria. Interestingly, stomaching yielded significantly different results for the salami and the meat paste. The inferior performance of stomaching for *Salmonella* extraction out of the meat paste may result from the very high reduction ratio and, thus, a very small meat particle size of the meat paste. The meat was spiked during the mincing process and *Salmonella*, therefore, sticks to the surface of these particles. In the coarsely ground salami, the meat particle size during the spiking procedure was much bigger and as a consequence, in comparison to the meat paste, the accessible surface area was much smaller. To transfer the pathogen to the dilution medium, it is necessary to bring the pathogen in contact with the liquid phase by homogenizing

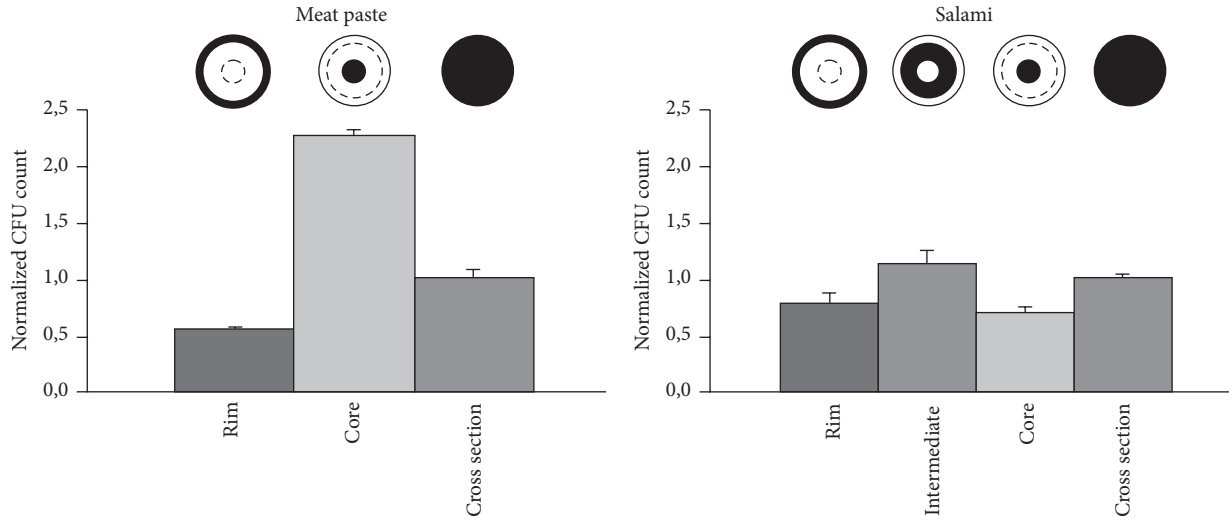

Figure 3: Pathogen distribution within meat paste and salami. The schematic drawings on top of the bars indicate the examined region of the sausages (black). Pathogen concentrations were determined after 8 min of FastPrep-24 treatment and are given in relation to the total concentration in whole cross sections.

the solid matrix. Consequently, harsh mechanical treatments like the FastPrep-24 system, which enable the rapid exchange between the particulate solid phase and the liquid phase, might be much more effective for finely minced sausages (e.g., meat paste) with a high particulate surface area, whereas, in case of more granular matrices with small overall surface areas, FastPrep-24 and stomaching perform similarly. In line with these considerations, a recent study with tomatoes, internally contaminated with *S. enterica*, showed that a short, but harsh, blending step was more effective than a longer stomaching treatment [19]. It would be interesting to evaluate whether suggested alternatives to stomaching like the Pulsifier, which was reported to perform equally for surface contamination while generating less debris [8, 9, 23], provide different results.

3.3. Analysis of Inner-Matrix Pathogen Distribution. Not only homogenization but also the region of sampling might influence the detection of a pathogen. Although the pathogen has been evenly distributed throughout the entire meat mass during the production process, it is possible that this even dispersal might change during the sausage ripening. Therefore, we divided the sausages into different regions, covering the outer rim, the core, and whole cross sections of the food products, and determined the concentration of *S. enterica* in each region (Figure 3). For homogenization, the FastPrep-24 treatment was chosen because this system had shown a good performance in both sausage types. The core of the meat paste contained up to fourfold higher concentrations of *Salmonella* than the outer rim, demonstrating a sharp gradient towards the center of the sausage matrix. In contrast, the distribution of *S. enterica* in the salami was relatively homogenous and no bacterial enrichment in the core was identified. However, the concentration of *Salmonella* was slightly increased within the intermediate region of this sausage type compared to rim and core. Further investigations are necessary to determine whether the pathogen distribution within the meat paste is based on the production process or on specific parameters like rigidity, water content, ingredients, or grain size of the meat.

Standard operation procedures are rather vague in terms of sample taking; in general they require "representative" samples, which can be interpreted as whole cross sections in case of sausages. However, our study shows that the assumption of a homogenous pathogen distribution within the matrix is not necessarily realistic. For the meat paste, we identified surprisingly high differences in pathogen load, presumably resulting from a prolonged survival in the sausage core, which might be a result of a different water activity or pH, both major factors for pathogen inactivation [24, 25]. Different types of sausages or in general any food product with a suspected inner-matrix contamination (thus including not only meat products but also fresh produce or dairy products) might have a characteristic distribution (and survival) pattern for a particular bacterial species, including the formation of bacterial aggregates [26–29]. Thus, it might be worthwhile to elucidate the pathogen distribution in other food products, since a more sophisticated and risk-based sampling of food regions with higher bacterial loads might enable better detection limits.

4. Conclusions

The results presented in this work demonstrate the pivotal role of sampling and homogenization for the reliable detection of pathogens in specific food products. It becomes evident that the general lack of precise advices regarding sample pretreatment might be responsible for considerable interlaboratory differences in pathogen detection. This not only is important for microbiological investigations, but also might be suitable as a general reference point for other whole-cell detection methods, for example, fluorescence

in situ hybridization [30]. However, choosing the appropriate homogenization device should consider not only the efficiency of detection, but also the ease of handling, costs, and high-throughput capabilities. Summing up, to enable improved pathogen detection methods, standardized and harmonized sample preparation protocols are needed for different food matrices.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

The Use of Plant Antimicrobial Compounds for Food Preservation

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Foodborne disease is a global issue with significant impact on human health. With the growing consumer demand for natural preservatives to replace chemical compounds, plant antimicrobial compounds must be thoroughly investigated for their potential to serve as biopreservatives. This review paper will focus on the plant-derived products as antimicrobial agents for use in food preservation and to control foodborne pathogens in foods. Structure, modes of action, stability, and resistance to these plant compounds will be discussed as well as their application in food industries and possible technologies by which they can be delivered. Benefits as well as challenges, such as the need for further research for implementation and governmental regulation, will be highlighted.

1. Introduction

The use of plants for healing dates to prehistory. As early as 60,000 years ago, the Neanderthals, in present-day Iraq, used plants including hollyhock for healing. These plants are still used globally [1]. Hippocrates wrote about several hundred medicinal plants in the late fifth century B.C. and the Bible mentions healing plants, such as frankincense and myrrh, which have antiseptic properties [1]. Plant oils and plant extracts have been utilized for thousands of years, serving many purposes, such as food preservatives and medical therapeutic agents [2]. The compounds that are found in some spices and produced by herbs act as self-defense mechanisms to protect the plant against infectious organisms [3]. They are also used by many cultures as flavoring agents and as natural preservatives in food. For example, in foods of India and in traditional Indian medicine, many spices, including garlic, black pepper, cumin, clove, ginger, and caraway, are used [4].

The majority of western plant pharmaceutical information was destroyed during the fall of ancient civilizations, but the Renaissance saw a revival in the use of medicinal plants in the western world [1]. In North America, indigenous cultures have used medicinal plants since prehistory and Americans of European origin began using botanicals in the 19th century to counter the toxic medical practices of that time, such as the use of mercury baths to treat syphilis [1].

Asian culture focuses on the use of herbs to treat diseases and illnesses. Throughout China's history, extensive research was conducted to learn the curative powers of plants. The *Imperial Grace Formulary* that was compiled in 985 C.E. contains 16,834 herbal entries. Indeed, Chinese medicine reflects traditions developed over 3,000 years and is a holistic approach that takes into account a condition in relation to the whole body in contrast to Western medicine that focuses on a specific cause and attempts to control it. Although Chinese and Western medicine are based on very different philosophies, Chinese herbal medicine has become well-known in the US and in Britain over the last few decades [5].

2. Approaches to Control Bacteria: Human Health and Food

Food safety is a global issue with significant implications for human health. The World Health Organization reports that, annually, unsafe food results in the illnesses of at least 2 billion people worldwide and can be deadly. Some countries have made great progress in controlling foodborne diseases,

but the number of those affected by foodborne diseases is growing globally (WHO, 2004). In the United States, the Centers for Disease Control and Prevention (CDC) estimate that each year about 1 in 6 Americans becomes ill and thousands die of foodborne diseases (http://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html).

Thermal processing is a common method of destroying vegetative microorganisms to ensure food safety, but this technique may cause undesirable nutritional and quality effects [3]. Preservatives are commonly used to reduce the risk of foodborne illnesses. Increasing regulatory restrictions and consumer negative response to chemical compounds and to the use of antibiotics in agriculture have contributed to the pressure for the development of alternative compounds for use as antimicrobial agents [6].

Antimicrobial agents have been predominantly isolated from bacteria and fungi and either produced through fermentation or produced chemically [1]. In the United States, one-quarter to one-half of pharmaceuticals are derived from plants, but very few are used as antimicrobials. Worldwide, spending on anti-infective agents has increased in recent years due to the limited effective lifespan of antibiotics as new resistant microbes emerge [1]. New sources, including plants, must be thoroughly investigated for identification of novel antimicrobial compounds. For example, it is known that some spices and herbs confer antimicrobial activity. Although there are conflicting reports in the literature about the absolute efficacy of various spices and herbs, Holley and Patel [7] state that the spices, cinnamon, mustard, vanillin, clove, and allspice, and some herbs, specifically oregano, rosemary, thyme, sage, and basil, all confer strong antimicrobial activity. They continue by stating that there are many others that show limited or moderate antimicrobial activity as well. However, Nychas [8] suggests that Gram-positive bacteria are generally more sensitive than Gram-negative bacteria to the antimicrobial compounds of spices.

Alternatives to traditional antimicrobial compounds include bacteriophages, antimicrobial phytochemicals, and antimicrobial peptides. There is less extensive research on the application of antimicrobial compounds from sources other than bacteria. This review paper will focus on the use of plant products as antimicrobial agents, specifically for use in food preservation and safety. Given the consumer demand for natural preservatives and the rapid rate of plant species extinction, it is imperative that more research is focused on the application of plant antimicrobials to food safety.

3. Plant Antimicrobial Peptides (pAMPs)

First discovered in 1942, antimicrobial peptides are produced by bacteria, animals, and plants to serve as natural defense compounds against pathogens. Although generally accepted to be small molecules, there is debate amongst researchers about their exact size. According to Joerger [6], they have a molecular mass between 1 and 5 kDa, but, according to Choon Koo et al. [9] and Garcia-Olmedo et al. [10], they range in size between 2 and 9 kDa. Furthermore, they are predominantly positively charged and are amphiphilic [11]. Although there are many sources of AMPs, this section will focus on plant-derived antimicrobial peptides and their modes of action.

Research clearly demonstrates that antimicrobial peptides are a key part of a plant's defense against pathogens, serving roles both in the defense response upon infection and as part of the preexisting defense barrier. Research has demonstrated that a peptide serves a defense role based on whether peptide-sensitive mutants of pathogens show decreased virulence in plant tissues containing the respective peptide and/or whether overexpression of the peptide results in enhanced tolerance of the plant to the pathogen [10]. Some peptides show specificity towards Gram-negative or Gram-positive bacteria, but most are able to inhibit the activity of both [12]. Plant AMPs are predominantly cysteine-rich compounds that have been isolated from different plant species and from different tissues. A recent review has listed more than twenty different pAMPs [13]. Although there is debate amongst researchers about the number of families, they can be divided into distinct protein families based on structure and amino acid sequence characteristics [9]. These plant antimicrobial peptide families are shown below.

3.1. Thionins. The first discovered pAMPs, thionins, are toxic to yeast, fungi, and Gram-positive and Gram-negative bacteria [14]. The example of wheat purothionin-α1 [15] is shown in Table 1 with 45 amino acids. They are able to induce leakage of intracellular material in bacteria and yeast. Regarding mode of action, it has been shown that they cause cell permeability to isoaminobutyric acid and affect electrical currents in artificial membranes. Purified genetic variants of thionins exhibited differences in activity and some differences in specificity [10]. López-Solanilla et al. [16] used an *in vitro* method to show that thionins purified from wheat flour have a strong inhibitory effect on multiple strains of *Listeria monocytogenes* and on a strain of *Listeria ivanovii*, which is another pathogenic species of the genus *Listeria*. The MICs (minimum inhibitory concentrations) are listed in Table 2. The authors also studied the effect of temperature on listerial susceptibility to AMPs and determined that a shift from environmental temperature (20°C) to mammalian host temperature (37°C) made *L. monocytogenes* more sensitive to thionin, but the opposite was shown for *L. ivanovii*.

3.2. Plant Defensins. Structurally related to insect and mammalian defensins [14], plant defensins are able to inhibit bacteria and fungi [10]. The high antifungal activity of plant defensins underscores the significance of fungal pathogens in the plant world, which differs from the high antibacterial activity of animal defensins [14]. Defensins have been identified in locations of first contact and entry by plant pathogens, including peripheral cell layers, xylem, and stomatal cells and in cells lining the substomatal cavity. They have been isolated from tubers, leaves, pods, seeds, and flowers [10]. Defensin gene expression can be developmentally regulated or influenced by external stimuli. Pea, tobacco, radish, and *Arabidopsis* have defensin genes that are expressed upon pathogen infection.

Table 1: Molecular structures of selected pAMPs.

pAMP	Amino acid (AA) sequence	Plant source	Reference
Thionin-α1	KSCCRSTLGRNCYNLCRARGAQ KLCAGVCRCKISSGLSCPKGFPK	*Triticum aestivum* (wheat)	[15]
IbAMP1	QWGRRCCGWGPGRRYCVRWC	*Impatiens balsamina*	[22]
Lipid transfer protein 2	AITCGQVSSALGPCAAYAKG SGTSPSAGCCSGVKRLAGLA RSTADKQATCRCLKSVAGA YNAGRAAGIPSRCGVSVPY TISASVDCSKIH	*Hordeum vulgare* (barley)	[10]
MBP1	153 AA repeats with 13 AA motif: SGKGTDSGSST(K/Q)D 8 AA motif: GSQGGQGG	*Arabidopsis thaliana*	[17, 25]
Hevein	EQCGRQAGGKLCPNNLCCSQWG WCGSTDEYCSPDHNCQSNCKD	*Hevea brasiliensis*	[26]
Snakin1	GSNFCDSKCKLRCSKAGLADR CLKYCGVCCEECKCVPSGTYG NKHECPCYRDKKNSKGKSKCP	*Solanum tuberosum* (potato)	[27]
Kalata B1	CGETCVGGTCNTPGCTCSWPV CTRNGLPV	*Oldenlandia affinis*	[28]

There are four defensin groups, which are classified by structural properties. Group I inhibits Gram-positive bacteria and fungi, group II inhibits fungi, group III inhibits Gram-positive bacteria and Gram-negative bacteria, and group IV inhibits Gram-positive bacteria, Gram-negative bacteria, and fungi. Importantly, there has been no reported toxicity of plant defensins to animal or plant cells [10] which is very significant from a food safety standpoint should these antimicrobials be leveraged as biopreservatives. The mode of action of antifungal defensins is potentially dependent on electrostatic interactions between hyphal membranes and peptides that cause a rapid Ca^{2+} influx and K^{+} efflux [17].

IbAMP1 from the seeds of *Impatiens balsamina* represents one of the smallest pAMPs with only 20 residues (Table 1) and two disulfide bonds. IbAMP1 has been shown to be active against fungi, Gram-positive bacteria, and Gram-negative bacteria at micromolar levels [18–21]. Wu et al. [22] have demonstrated the concentration-dependent effect of Ib-AMP1 on the cell membrane of Shiga toxin-producing *E. coli* O157:H7. They showed that Ib-AMP1 exerted its bactericidal activity by interfering with outer and inner membrane integrity permitting efflux of ATP and interfering with intracellular biosynthesis of DNA, RNA, and protein [22].

López-Solanilla et al. [16] used an *in vitro* method that showed that potato defensin was only weakly inhibitory to *L. monocytogenes* and *L. ivanovii* at 37°C (MICs are listed in Table 2). However, at 20°C, the two species were resistant. Potentially, this shows an adaptive technique by *L. monocytogenes* and *L. ivanovii* to improve survival when in their primary natural habitat of decaying plant matter-filled soil, which is about 20°C.

3.3. Lipid Transfer Proteins (LTPs). These peptides were once thought to be involved in the transfer of lipids between organelles but have been shown to be involved in plant defense. They seem to have an important role in pathogen defense as well as during low temperature and salt stress. LTPs and defensins can synergistically inhibit fungal and bacterial growth in plants [17]. They are expressed in many areas of a plant, especially in exposed surfaces and in vascular tissues. LTPs have been isolated from barley (Table 1) [10], maize, spinach, *Arabidopsis*, broccoli, and radish and have demonstrated some specificity [10].

There is limited research on the mode of action of plant LTPs. *In vitro* research suggests plant LTPs function in plant defense against pathogens based on their ability to inhibit microbial growth. However, there is little direct evidence of the basis of their antimicrobial activity and, unlike other AMPs, they are thought to have many other roles *in vivo*. Ha-AP10, a LTP, completely inhibits the germination of spores at a concentration of 40 μg/mL. Regente et al. [23] demonstrated that Ha-AP10 acts as a fungicidal compound by not only inducing liposome leakage but also modifying the permeability in *Fusarium solani* spores. Although other factors may also contribute to the fungicidal activity, the membrane permeabilization mechanism is common to other pAMPs. In addition, the authors demonstrated that Ha-AP10 was able to permeabilize fungal cells in media containing 1 mM $CaCl_2$. This is significant because it more closely represents environmental conditions since the physiological concentration of free Ca^{2+} is 0.1–1 mM in the apoplast, where the fungal-plant contact is likely to occur. Furthermore, the authors demonstrated the selective toxicity of Ha-AP10 for fungal cells over plant (potato host cells). By conducting a follow-up experiment that used model membranes with encapsulated fluorescent probes, the authors hypothesized that this differentiation was due to the composition of phospholipids in the plant and fungal membranes. The activity of Ha-AP10, a cationic and hydrophobic peptide, may therefore be mediated by its electrostatic interaction with anionic membrane phospholipids [23].

Table 2: MIC values of antimicrobial agents.

AMP agent	Bacterial target	MIC	Recorded condition	Reference
Potato defensin	*L. monocytogenes*	>25 μg/mL	24 h at 37°C	[14]
Thionins	*L. monocytogenes*	2 μg/mL	24 h at 37°C	[14]
Snakin	*L. monocytogenes*	10 μg/mL	24 h at 37°C	[14]
Lipid transfer protein	*L. monocytogenes*	no effect	24 h at 37°C	[14]
Potato defensin	*L. ivanovii*	>25 μg/mL	24 h at 37°C	[14]
Thionins	*L. ivanovii*	5 μg/mL	24 h at 37°C	[14]
Snakin	*L. ivanovii*	10 μg/mL	24 h at 37°C	[14]
Lipid transfer protein	*L. ivanovii*	>25 μg/mL	24 h at 37°C	[14]
Carvacrol	*Salm.* Typhimurium	1 m/mol	16 h at 37°C	[34]
Cinnamic acid	*Salm.* Typhimurium	7.5 m/mol	16 h at 37°C	[34]
Diacetyl	*Salm.* Typhimurium	12.5 m/mol	16 h at 37°C	[34]
Eugenol	*Salm.* Typhimurium	3.0 m/mol	16 h at 37°C	[34]
Thymol	*Salm.* Typhimurium	1.0 m/mol	16 h at 37°C	[34]
Carvacrol	*E. coli*	1.5 m/mol	16 h at 37°C	[34]
Cinnamic acid	*E. coli*	5.0 m/mol	16 h at 37°C	[34]
Diacetyl	*E. coli*	7.5 m/mol	16 h at 37°C	[34]
Eugenol	*E. coli*	2.5 m/mol	16 h at 37°C	[34]
Thymol	*E. coli*	1.2 m/mol	16 h at 37°C	[34]
Thymol	*E. coli*	250 ppm	48 h at 37°C	[35]
Carvacrol	*E. coli*	375 ppm	48 h at 37°C	[35]
Cyclotide CyO2	*E. coli*	2.2 μM	37°C	[20]
Cyclotide Kalata B1	*E. coli*	≥100 μM	37°C	[20]
Cyclotide Kalata B2	*E. coli*	≥35 μM	37°C	[20]
Cyclotide Vaby A	*E. coli*	32.5 μM	37°C	[20]
Cyclotide Vaby D	*E. coli*	50 μM	37°C	[20]
Cyclotide CyO2	*S. aureus*	>50 μM	37°C	[20]
Cyclotide Kalata B1	*S. aureus*	>100 μM	37°C	[20]
Cyclotide Kalata B2	*S. aureus*	35 μM	37°C	[20]
Cyclotide Vaby A	*S. aureus*	>90 μM	37°C	[20]
Cyclotide Vaby D	*S. aureus*	>90 μM	37°C	[20]
Thymol	*S. aureus*	250 ppm	48 h at 37°C	[35]
Carvacrol	*S. aureus*	225 ppm	48 h at 37°C	[35]
Cyclotide CyO2	*S. enterica*	8.75 μM	37°C	[20]
Cyclotide Kalata B1	*S. enterica*	>100 μM	37°C	[20]
Cyclotide Kalata B2	*S. enterica*	>35 μM	37°C	[20]
Cyclotide Vaby A	*S. enterica*	90 μM	37°C	[20]
Cyclotide Vaby D	*S. enterica*	>90 μM	37°C	[20]

3.4. Myrosinase-Binding Proteins (MBPs). Myrosinase (EC 3.2.3.1) is a glucosinolate-degrading enzyme mainly found in the Brassicaceae special idioblasts, myrosin cells [24]. MBPs are involved in plant development and defense activities, primarily against pathogens and insects. The amino acid features of the MBP1 from Arabidopsis are shown in Table 1 [17, 25]. It has been discovered that the potential MBP mode of action is to act as ionophores over microbial membranes [17]. The mechanism of action has been proposed by Capella et al. [25] to be the hydrolysis of glucosinolates by myrosinase enzymes producing molecules with diverse modes of action against fungi, bacteria, and insects.

3.5. Hevein- and Knottin-Like Peptides. Both of these peptides inhibit fungi and Gram-positive bacteria *in vitro*. They have been primarily isolated from seeds, but hevein-like peptides (HTPs) have been found in other tissues as well [10]. Hevein is a small chitin-binding peptide that was initially isolated from rubber latex (Table 1) [26]. This peptide can be used as a fungicide but it may be allergenic, presenting

a significant barrier from a safety and labeling standpoint should this peptide be considered as a food preservative. The mechanism of action of HTPs is hyphal penetration that leads to cell burst [17].

3.6. Snakins. Snakins are antimicrobial peptides that have been isolated from potatoes (Table 1) [27]. The snakin-1 peptide is active against fungi and Gram-positive and Gram-negative bacteria at concentrations less than 10 μM [10]. The peptide is able to aggregate bacteria *in vitro* but does not mediate leakage or aggregation of artificial liposomes at low- or high-salt concentrations and does not destroy lipid membranes [17]. The mechanism of action of this peptide has yet to be elucidated.

Using an *in vitro* method, López-Solanilla et al. [16] showed that a snakin peptide had a strong inhibitory effect on *L. monocytogenes* and *L. ivanovii* (MICs are listed in Table 2). The *Listeria* species exhibited differential susceptibility to various pAMPs, which could be potentially linked to the differential fate of *Listeria* in different areas of the plant.

3.7. Cyclotides. Plant cyclotides do not have N- or C-termini because this peptide has a cyclic structure, which serves an important role in the peptide's activity and stability. The structure is a head-to-tail backbone with six conserved cysteine residues, forming a knot motif. They can be classified into two subfamilies: Mobius and bracelet. Mobius cyclotides have a twist formation in the backbone whereas bracelet cyclotides do not have a twist. A third group in the cyclotide family consists of proteinase inhibitors that were isolated from *Momordica cochinchinensis,* and another cyclotide structure, kalata B8, isolated from *Oldenlandia affinis,* seems to be a hybrid of the Mobius and bracelet subfamilies [28]. Kalata B1 is listed in Table 1 [28].

These peptides serve various plant defensive roles, including cytotoxicity to plant tumor cells, antiviral and insecticidal activities, and proteinase inhibition. Some also exhibit antibacterial activity, with peptide kalata B1 and circulin A active against Gram-positive bacteria, such as *Staphylococcus aureus*, and circulin B and cyclopsychotride active against both Gram-positive and Gram-negative bacteria [28]. Pränting et al. [29] conducted research to determine the antibacterial activity of various cyclotides against several Gram-positive and Gram-negative bacterial strains (MIC values are listed in Table 1). From the five evaluated cyclotides, cycloviolacin O2 (cyO2) was determined to be the most potent antibacterial cyclotide, showing high efficacy against Gram-negative bacterial species, including *E. coli*. This activity is significant given the evidence that other antimicrobial agents, such as nisin, are generally less active against Gram-negative species. However, the mode(s) of action for cyclotides have not yet been elucidated. More research is needed to determine mechanism(s) of action and further biological functions of cyclotides, but these compounds have great potential to serve as novel antibiotics and antiviral therapies to control infectious diseases [28].

3.8. Peptides from Hydrolysates. Plant protein hydrolysates can be a source of bioactive peptides [13]. Hydrolysis is either done enzymatically or by acids. Hydrolysates from leguminous plants are particularly favored as they are parts of the food ingredients for many countries in the world [13]. As summarized by Salas et al. [13], the enzymatic hydrolysates from common bean varieties of *Phaseolus* demonstrated antimicrobial activities against *S. aureus* and *Shigella flexneri* with MIC values in the range of 0.1 to 0.99 mg/mL. Another report has shown that the alcalase hydrolysates of rapeseed (*Brassica napus*) protein inhibited the protease activity of human immunodeficiency virus (HIV) that was expressed in *E. coli* cells [30].

Some industry by-products represent another source of bioactive peptides that possess antimicrobial properties. For example, the palm kernel expeller (PKE) is produced after palm kernel oil production [31]. Tan et al. tested the efficacy of the purified alcalase- and tryptic-hydrolysates of PKE, PAH, and PTH on *Bacillus cereus* [31, 32]. It was shown that both PAH and PTH disrupted the membrane integrity of *B. cereus*, allowing efflux of K^+, depleted the ATP molecules, and inhibited the intracellular macromolecule metabolism especially the RNA of the bacterium.

4. Structure of pAMPs Related to Their Modes of Action

Plant AMPs have similar physical properties but diverse primary amino acid sequences. In addition, pAMPs have a range of secondary structures, but there are at least four major themes: loop structures, amphiphilic peptides with two to four β-strands, amphipathic α-helices, and extended structures [11]. However, there are peptides that do not fit into this structure classification, such as many bacterially produced peptides that have two domains, one of which is α-helical and the other of which has a β structure. In addition, there is little scientific literature describing the tertiary structures of pAMPs. However, *in silico* analyses have shown that pAMPs have similarities in their three-dimensional structures [12].

The antibacterial mode of action for most pAMPs involves cell membranes of targeted organisms and is driven by net positive charge, flexibility, and hydrophobicity to enable interaction with bacterial membranes [11]. Although it was originally thought that the sole mode of action was permeabilization of the bacterial cell membrane, research suggests there may be alternative modes of action or that the pAMPs act upon multiple cell targets. However, interaction with the bacterial cell membrane is critical. There are several models in the literature that illustrate interaction at the cell membrane, each of which uses a different intermediate that leads to either formation of a transient channel, translocation across the membrane, or micellization or dissolution of the membrane. The modes of action are therefore either membrane acting (permeabilizing) or nonmembrane acting (nonpermeabilizing) since translocation does not cause membrane disruption but allows entrance to target essential intracellular processes. In addition, a peptide may target both the cell membrane and intracellular components [11].

The antifungal mode of action was first thought to only involve cell lysis or interference with the synthesis of

the fungal cell wall. However, research indicates additional modes of action, including permeabilization, binding to ergosterol/cholesterol in the membrane, depolymerization of the actin cytoskeleton, and targeting intracellular organelles, such as mitochondria [11]. The mode of action of some antifungal peptides is still debated amongst researchers. Plant antimicrobial peptides with predominantly antifungal efficacy tend to be rich in polar and neutral amino acids. The mode of action by plant defensins against fungi has recently been reviewed by Vriens et al. [33].

Antiviral activity is often related to a direct effect on the viral envelope or related to the viral adsorption and entry process [11]. Some antiprotozoal modes of action are similar to antibacterial, antifungal, and antiviral mode of action, such as cell membrane disruption via pore formation or direct interaction with the lipid bilayer. However, there are conflicting reports found in the literature which indicate that antiprotozoal activity may be dependent on peptides that are different from viral, fungal, and bacterial activities [11].

5. Resistance to pAMPs

Microbial resistance to antimicrobial agents used in food preservation and sanitation is a major concern. Antibiotic resistance is generally caused by transfer of resistance genes between bacterial cells [34]. Laboratory and clinical studies have determined that resistance to AMPs is less likely than resistance to conventional antibiotics [35]. This is likely due to their membrane-targeting mechanism of action that is more difficult to develop resistance to antibiotics, which generally target macromolecular synthesis (DNA, RNA, and protein) [36]. However, it has been demonstrated that specific genes can confer resistance to pAMPs. For example, the *pagP* gene increases resistance to the bactericidal effects of some antimicrobial peptides in *Salmonella* [37]. Changes to the targeted organism's cell membrane may also lead to resistance. It has been difficult to develop resistant strains from previously sensitive strains to particular antimicrobial peptides [6]. This is an ongoing area of research.

It is imperative to note that commensal bacteria, which are beneficial to the host organism, such as *Lactobacillus* in the intestines of humans, are relatively resistant to the action of endogenous antimicrobial peptides [35]. This type of resistance and AMP specificity suggests that pAMPs could potentially be used in food application after further toxicology studies are conducted.

6. Phytochemicals

Phytochemicals are nonnutritive plant components that confer organoleptic properties and serve as antimicrobial agents. The concentration, composition, structure, and functional groups serve an important role in determining antimicrobial activity. Phenolic compounds are generally the most effective [7]. Based on their chemical structures, they may be divided into different categories including simple phenolic compounds, flavonoids, quinones, tannins, and coumarins. The most important phytochemicals used as food preservatives are essential oils, which have been used by humans across the continents since ancient times. Some alkaloids from plants have also been used as antimicrobials in food. Recently, many different phytochemicals have been listed by Negi [38] and their antibacterial activities have been summarized. The antifungal and antifungal toxin activities from various plant extracts including phenolic compounds and essential oils have also been recently reviewed [39]. Polyphenolic compounds from fruits such as cranberry, pomegranate, blueberry, raspberry, and grape were also summarized in 2014 for their antiviral activities against human enteric viruses [40]. Here we briefly review these phytochemicals for their antimicrobial activities in food applications with some examples.

6.1. Simple Phenolic Compounds. Simple bioactive phytochemicals are comprised of a single substituted phenolic ring [41]. There is some evidence that the sites and number of hydroxyl groups on the phenolic ring are related to the degree of toxicity to microorganisms, with increased hydroxylation resulting in increased toxicity. It has also been suggested that higher oxidation confers greater inhibition [1]. The mode of action is enzyme inhibition by the oxidized compounds. Phenolic compounds are known to alter microbial cellular permeability, resulting in loss of macromolecules, and interact with membrane proteins, causing structural changes [39]. A simple phenol example is caffeic acid, which is found in thyme and tarragon and is active against fungi, viruses, and bacteria. Eugenol is a phenolic compound found in clove oil that is active against bacteria and fungi [1].

6.2. Flavones, Flavonols, and Flavonoids. Flavones are phenolic compounds with one carbonyl. Flavonols are phenolic compounds with a carbonyl and a 3-hydroxyl group. Flavonoids are hydroxylated phenolic structures with a C3–C6 aromatic ring linkage. They are effective against many microorganisms because of their ability to bind to and inactivate proteins and to complex with bacterial cell walls. Catechins provide the antimicrobial activity in oolong teas. The green tea polyphenol, epigallocatechin-3-gallate, was shown to be antiviral against hepatitis B virus replication *in vitro* [42]. Unlike simple phenolic compounds, the degree of hydroxylation does not predict the level of toxicity to microorganisms [1].

6.3. Quinones. Quinones are aromatic rings with two carbonyls, providing a stable source of free radicals. In addition to serving as antioxidants, they are potent antimicrobial compounds. Similar to flavones, flavonols, and flavonoids, the antimicrobial mode of action is to bind to and inactivate proteins. In addition, they may make substrates unavailable to the microorganism [1]. The 6-(4, 7-dihydroxy-heptyl) quinone isolated from the leaves of *Pergularia daemia* (Forsk.), a traditional medicinal plant, was shown to be effective against several food-contaminating pathogens including *Bacillus subtilis, Staphylococcus aureus*, and *Escherichia coli* [43].

6.4. Tannins and Coumarins. Tannins are polymeric phenolic substances that are divided into hydrolysable and

condensed tannins (also known as proanthocyanidins). The latter are based on flavonoid monomers and hydrolysable tannins are based on gallic acid. Tannins may be formed by polymerization of quinones or by condensation of flavan derivatives. Their antimicrobial mode of action is similar to that of quinones and they have been shown to be toxic to bacteria, yeasts, and some fungi [1]. Tannins naturally occur in many fruits, nuts, and seeds. A recent review by Lipińska et al. [44] shows that the hydrolysable ellagitannins found in pomegranate, strawberry, blackberry, raspberry, walnuts, almonds, and seeds exhibit antimicrobial activity against fungi, viruses, and, importantly, bacteria, including antibiotic-resistant strains such as methicillin-resistant *Staphylococcus aureus* (MRSA). Additionally, a recent article has comprehensively reviewed the antimicrobial activities of bioactive components from berries including flavonoids (anthocyanins, flavonols, and catechins), phenolic acids, stilbenes, and tannins [45].

Coumarins are phenolic structures comprised of a fused benzene and alpha-pyrone ring. Although toxic to some animals, they have been shown to have species-dependent metabolism, with toxic coumarin derivatives excreted in human urine without adverse health effects. A recent review summarizes not only the anti-inflammatory, anticoagulant, anticancer, antihypertensive, antitubercular, anticonvulsant, antiadipogenic, antihyperglycemic, antioxidant, and neuroprotective properties of coumarins but also their antibacterial, antifungal, and antiviral activities [46].

6.5. Essential Oils. Essential oils, or terpenes, are secondary metabolites and are based on an isoprene structure. They are volatile compounds that provide the fragrance of plants and are mainly responsible for the flavor and aroma of spices. Terpenoids contain additional elements, such as oxygen, and confer activity against bacteria, fungi, protozoa, and viruses [1]. Research has shown that essential oils have anti-inflammatory, bactericidal, antiviral, and anticancer effects and possess antioxidant activity [47]. For example, Delaquis et al. [48] determined that the essential oil of cilantro was particularly effective against *Listeria monocytogenes,* potentially because of long chain alcohols and aldehydes since the antimicrobial properties of alcohols are known to increase with molecular weight. A review by Seow et al. [49] has included 47 different essential oils as antimicrobials. The antibacterial and antifungal activities of these essential oils have been listed. As essential oils contain highly diverse groups of phytochemicals, their antimicrobial modes of action have been suggested to involve multiple targets. The unique hydrophobicity features of essential oils render their abilities to react with lipids on the bacterial cell membranes, increasing the membrane permeability and disturbing the original cell structure [50, 51]. The clover essential oil has been shown to cause an extensive lesion of fungal cell membrane [52]. Essential oil has also been shown to inhibit viral protein synthesis at multiple stages of viral infection and replication [53].

6.6. Alkaloids. Alkaloids are heterocyclic nitrogen compounds and have demonstrated limited microbicidal activity as well as possessing an antidiarrheal activity. An example of an alkaloid is berberine [1]. Berberine is the main antibacterial substance of rhizoma Coptidis (*Coptis chinensis* Franch) and cortex Phellodendri (*Phellodendron amurense* Ruprecht) [54]. The MICs of berberine against methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria ranged from 32 to 128 μg/mL. Ninety percent inhibition of MRSA was obtained with 64 μg/mL or less of berberine.

7. Stability of Plant Antimicrobial Compounds

The effect of food processing on plant-derived antimicrobial compounds must be evaluated. Plant-derived foods are often exposed to acidic or alkali conditions or high heat during processing to destroy microorganisms to enhance microbial food safety. The aforementioned conditions are used to peel fruits and vegetables and to recover proteins from cereals and legumes [55]. However, these conditions may destroy natural plant antimicrobial compounds, which serve as defense mechanisms to the plant and have potential to serve as natural antimicrobial compounds against human pathogens.

Research by Friedman and Jürgens [55] indicated that the chemical structure of phenolic compounds has a significant effect on their susceptibility to destruction at alkali conditions. By using ultraviolet spectroscopy, they determined that gallic acid, caffeic acid, and chlorogenic acid were not stable in high pH and that the spectral transformations were not reversible when the pH was reduced back to neutral conditions. The phenolic OH groups were hypothesized to be primarily responsible for the spectral changes since ferulic acid (with one OH group) was more stable in high pH versus caffeic acid (with two OH groups) and gallic acid (with three OH groups). Furthermore, the ionized and resonance forms of multiring structures conferred more resistance to high pH versus monoring structures. Therefore, multiring catechin, epigallocatechin, and rutin had less spectral transformations at high pH conditions versus monoring gallic acid, caffeic acid, and chlorogenic acid. This research provides a foundation that suggests structural elements that may confer stability at alkali conditions. It is critical to determine whether natural antimicrobial compounds are stable in food processing conditions in order to determine their feasibility as alternative antimicrobial compounds to foodborne pathogens in food systems.

In addition to this foundational research under artificial conditions, it is critical for natural antimicrobials to be tested in food systems. For example, solubility and food constituents, such as proteins and lipids, could potentially impact efficacy and stability of plant-derived antimicrobial compounds. Research conducted by Aureli et al. [56] indicated that thyme essential oil reduced viable *Listeria monocytogenes* L28 cells in a meat matrix but noted that there was a decreased efficacy in the food system compared to *in vitro* testing. Pränting et al. [29] reported that the antibacterial efficacy of the pAMP cyclotide cyO2 was reduced in media containing salt, which suggests that this peptide would be less efficacious as a preservative in a high-salt food. The authors do note, however, that even though some research has indicated similar influence of media composition on

antibacterial efficacy of AMPs, the AMPs were still active against bacteria in biological systems. It is essential that the antimicrobial compounds effectively reduce pathogenic bacteria to allowable limits or completely inactivate pathogens.

Because pasteurization can affect organoleptic and nutritional properties and increase processing costs and postpasteurization contamination can occur, Friedman and Jürgens [55] conducted a study to assess the stability of a naturally occurring antimicrobial, chlorogenic acid, in apple juice. The authors determined that the phenolic compound, chlorogenic acid, was stable in the low pH, heat treatment, and storage of apple juice, offering a promising candidate to combat contamination by *E. coli* O157:H7 and *Salmonella* Typhimurium of nonfermented apple juice products. Payne et al. [57] determined that the phenolic compounds propyl paraben (propyl ester of p-hydroxybenzoic acid) and tertiary butylhydroquinone (TBHQ) were significantly more effective than potassium sorbate, a commonly used antimicrobial, against *Listeria monocytogenes* in a model milk system containing 10% nonfat milk solids at 35°C. However, TBHQ was inconsistent in its activity. Although the authors suggest that this study indicates that inhibition would be achieved at refrigeration temperatures, it seems that there may have been an error in conversion of Celsius to Fahrenheit, since 35°C is much greater than refrigeration temperatures. The authors do raise a valid point because it is important to conduct studies at the proper storage temperature of the food product in order to mimic normal food shelf conditions and because temperature greatly affects the survival rate of foodborne pathogens.

8. Applications of Plant Antimicrobial Compounds in Food Industry

For the food industry, plant antimicrobial compounds have potential use as biopreservatives and bioinsecticides, with potential use for development of genetically modified crop plants with increased disease resistance as well as use against foodborne pathogens. In fact, research has already shown the effectiveness of plant compounds against virulent foodborne pathogens. For example, essential oils have been used to help control *Listeria monocytogenes.* Aureli et al. [56] used a paper disc diffusion method to demonstrate that 12 out of 32 essential oils were active against *Listeria monocytogenes*, with clove, cinnamon, pimento, origanum, and thyme showing the greatest inhibition. However, the application of plant antimicrobial compounds for controlling growth of foodborne pathogens must incorporate the range of activity against the microorganisms of concern associated with a particular product. For example, essential oils are typically more effective against Gram-positive than Gram-negative bacteria. But some, such as clove and cinnamon, have been shown to be effective against both [3]. Olasupo et al. [58] demonstrated that 5 natural organic compounds were effective against the Gram-negative bacteria *E. coli* and *Salmonella* Typhimurium with efficacy in the following order: thymol > carvacrol > eugenol > cinnamic acid > diacetyl. Table 1 shows the MIC values of various antimicrobial agents against pathogenic bacterial targets at various conditions. This table illustrates that the efficacy of natural antimicrobials is impacted by environmental conditions and antimicrobial agents have a wide range of activity against bacterial targets. This information must be fully verified for a particular food product when considering the use of plant-derived antimicrobials in the food product. Although this review focused on efficacy against pathogenic organisms, it should be noted that the common spoilage bacteria, *Pseudomonas*, are generally resistant to plant antimicrobials due to the production of exopolysaccharide layers that offer protection and delay penetration of antimicrobials [3].

Another potential use of antimicrobial peptides in the food industry is replacement of antibiotics used in animal production to increase feed efficiency. Although there are some conflicting reports in the literature, Jin et al. [59] determined that pigs fed increasing levels (0 to 600 ppm) of refined potato protein (RPP) from *Solanum tuberosum* L. cv. Gogu Valley demonstrated linear improvements in performance and a linear decrease in fecal and intestinal bacteria, suggesting that RPP was effective at higher levels. Since the potato tubers of Gogu Valley are known to contain the AMP potamin-1 (PT-1), the authors suggest that the AMP may have caused the decrease in microbe numbers, contributing towards increased performance. Given the linear effects demonstrated with increasing levels of RPP in the pigs' diets, future studies that evaluate higher levels of RPP in pigs (as well as other production animals) should be completed to more fully elucidate the potential of RPP as an alternative to antibiotics. Furthermore, other AMPs should be evaluated as potential performance enhancers and chemical modification or encapsulation methods should be evaluated to prevent degradation by proteolytic enzymes within the digestive system of production animals [6]. However, it is known that cyclotides are stable against proteases, such as pepsin and trypsin, and their cyclic structure confers protection against exopeptidases [29].

9. Technologies by Which Plant Antimicrobials Can Be Delivered

There are various methods by which plant antimicrobials could be delivered. The most suitable methods must incorporate cost-effectiveness, stability, and efficacy of the compound under processing, transportation, and storage conditions. A recent technology incorporates antimicrobials into packaging materials instead of the food itself. This technique offers the advantage of concentrating the antimicrobial at the surface of the food product, which is where potential pathogens grow, and reduces obstruction from food particles [7].

Microencapsulated antimicrobial agents incorporated in food packaging have been demonstrated to successfully destroy a range of microorganisms, offering a controlled-release preservation technique. This application is a type of active packaging (AP), in which the conditions of the packaged food are changed to better preserve the sensory attributes, safety, and shelf-life of the product. Since microbial contact primarily occurs on the surface of a packaged

food, antimicrobial activity should be focused on solid or semisolid surfaces by either indirect contact using antimicrobial volatiles or via direct contact between the antimicrobial package and the food surface. Research has successfully incorporated antimicrobial peptides, such as bacteriocins, as well as phytochemicals, such as essential oils, into packaging materials [60].

Guarda et al. [60] demonstrated the antimicrobial activity of microencapsulated carvacrol and its isomer thymol, which are phenolic compounds that are major components of essential oils with known antimicrobial activity. By creating emulsions with the compounds and applying them to a polymer film, the authors demonstrated their antimicrobial activity by the agar plate method. The minimal inhibitory concentration (MIC) of thymol was 125–250 ppm and the MIC of carvacrol was 75–375 ppm against both pathogenic and nonpathogenic microorganisms: *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Listeria innocua*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. The highest synergism was determined to be 50% of each compound and various concentrations were studied to determine the required concentration for the most resistant microorganism, *E. coli*, based on zones of inhibition. The authors found similar findings to others in the literature that Gram-positive bacteria were more sensitive than Gram-negative bacteria, such as *E. coli*, potentially due to the outer membrane that surrounds the lipopolysaccharide wall inhibiting the penetration of the hydrophobic phenolic compounds. The required concentration of the antimicrobial agent to confer antimicrobial activity therefore depends on the type of targeted organism in a given food product. Furthermore, it is important to note that loss of antimicrobial activity may occur during preparation of the packaging. Lower losses were observed in the coating process utilized [60] than high-temperature processes, such as extrusion blow-molding. The authors noted that the release rate of the microcapsules was lower and more controlled as compared to films with antimicrobial agents directly incorporated into the matrix, potentially due to the affinity of the carrier, gum arabic, to a polar compound and the good film forming capability of gum arabic. This research provides a strong foundation for the use of these GRAS approved materials in active packaging, but more research is required on the use of this packaging on food systems.

Chitosan is a hydrophilic polymer that is obtained commercially by N-deacetylation of chitin. Chitosan exhibits some antimicrobial activity against fungi, algae, and some bacteria. Antimicrobial efficacy is influenced by the type of chitosan, molecular weight, and environmental conditions. Chitosan is limited by its insolubility in water, high viscosity, tendency to coagulate with proteins, and poor solubility at high pH. However, water-soluble salts can be formed by neutralization with acids. The exact antimicrobial action of chitosan is debated based on review of the literature, but it has been suggested that chitosan interacts with negatively charged microbial cell membranes, leading to cellular leakage. The polymer also acts as a chelating agent that binds trace metals, inhibiting toxin production and microbial growth. Research is also being conducted to determine its ability to elicit natural plant defenses when applied to plant tissues or cultured plant cells [61].

Chitosan is a biopolymer that is safe for human consumption and has several effective delivery methods, including the use as a seed treatment and as an edible antifungal coating material for postharvest produce. Chitosan films are semipermeable, durable, long-lasting, natural, inexpensive, and nontoxic and have been successfully used to delay decay of various fruits and vegetables potentially due to decreased rates of respiration, delay of ripening from the reduction of ethylene and carbon dioxide production, and fungal inhibition. Additional antimicrobial agents can be applied to chitosan films so that they serve as an active type of packaging, releasing the biopreservatives in a controlled manner onto the food surface to inhibit microbial growth. N,O-Carboxymethyl chitin films have been approved for use in fruits in both USA and Canada. Further research is needed to determine novel derivatives of chitosan with increased antimicrobial activity [61].

Plant antimicrobials can be delivered via plant extracts or consumed whole. The literature generally cites that spice extracts are less antimicrobial than the whole spice. For example, Lachowicz et al. [62] used an agar well diffusion method to demonstrate that essential oils extracted from five different varieties of *Ocimum basilicum* L. plants showed equivalent or better antimicrobial activity against a range of Gram-positive and Gram-negative bacteria, yeasts, and molds compared to the purified components linalool and methyl chavicol either separately or together. In contrast, Delaquis et al. [48] determined that fractions of dill and cilantro oil had greater antimicrobial activity than the whole oil. The latter authors aptly suggest that essential oils, which are naturally variable in composition, could more reliably be used as preservative agents if the concentrations of antimicrobial components could be adjusted to consistent levels for the needed spectrum and strength of microbial inhibition. More research is required in this area.

10. Regulation of Plant-Derived Antimicrobials for Food Application

The exact number of plant-derived antimicrobials approved for food application globally is difficult to discern due to the limited amount of information available. Food additives are closely controlled by legislation worldwide, but there is little agreement between countries regarding food additives that are safe, permitted concentrations, and specific permitted food uses [63]. In the US, the Food and Drug Administration (FDA) evaluates the safety of unapproved food additives to determine whether they should be approved. The evaluation includes the amount of the substance that would normally be consumed as well as short- and long-term health effects and other safety considerations. When a food additive is approved, the FDA issues a regulation that may include the types of foods in which it can be used, the maximum amount allowed, and its proper identification on food labels. These regulations are published in the Title 21 of the *Code of Federal Regulations*. According to the FDA's

Guidance for Industry: Antimicrobial Food Additives, the FDA has regulatory authority over food additives. However, the Environmental Protection Agency (EPA) sets tolerances for pesticide chemicals and pesticide chemical residues in or on foods, which are enforced by the FDA. It should be noted that antimicrobials applied to, or included in, food packaging materials are excluded from the definition of "*pesticide chemical*" and thus are regulated as food additives by FDA.

Although regulation varies worldwide, other countries have similar departments or agencies in place to evaluate the safety and provide guidance and regulation on food additives. In Europe, there are relatively few compounds that are allowed as food preservatives, and these are primarily organic acids [63]. There is a strict protocol in place in order for food additives to be approved for human consumption. When applying for authorization of a new food additive, an applicant submits a formal request to the European Commission, which is the executive body of the European Union, and includes information on the substance, including scientific data concerning safety. Upon acceptance of the application, the Commission requests the European Food Safety Authority (EFSA) to issue an opinion on the safety of the substance for its intended uses. In addition to carrying out safety evaluations of new food additives before they can be authorized for use in the European Union (EU), EFSA reviews certain food additives that have new scientific information and/or changing conditions (http://www.efsa.europa.eu/en/topics/topic/additives.htm). Similarly, the Chinese government employs the China Food Additives Association (CFAA) as the only registered nationwide food additive and ingredients industry organization to make evaluations and assist the government in making regulations in the food and food additives industry (http://www.cfaa.cn/english.htm).

Given the strict regulation and the relatively limited *in vivo* research on pAMPs, a literature review and a search of the web pages of the governmental agencies listed above yielded no known approved plant AMPs in those countries for food application. The agencies are constantly evaluating new potential food additives, including biopreservatives, so regulation of plant-derived antimicrobials can be expected to be seen in the future.

11. Conclusion

Food processing and some preservation techniques, such as heating, may alter food's nutritional or organoleptic properties. Microbial resistance to current antimicrobial compounds has increased in recent years worldwide; therefore, alternative compounds must be investigated and developed. This review has cited many of the benefits and lists some of the current hurdles of implementing plant-derived antimicrobials in food application. Most significantly, despite extensive *in vitro* research of plant-derived antimicrobials, there are limited *in vivo* studies, yielding knowledge about the toxicology of the extensive repertoire of compounds. Future toxicology research will aid the governmental food safety agencies in their evaluation and regulation of these compounds for food application.

Because of variation in stability and efficacy to various food processing parameters and food systems, it is critical that plant-derived antimicrobials be selected and delivered so that they are active against potential pathogens in particular food and are stable throughout the food's shelf life. Effects of these compounds in combination with other compounds or techniques must be more thoroughly investigated. For example, hevein- and knottin-like peptides are active against Gram-positive bacteria and fungi. However, research should be conducted to verify if they would also be active against Gram-negative bacteria if chelators that perturb the outer membrane of Gram-negative bacteria were also present. In addition to these scientific evaluations, sensory studies must be conducted to ensure that the organoleptic properties of a food are not impacted by the natural antimicrobial peptides.

Given the consumer demand for more natural products and the growing need for alternative preservatives to ensure food safety, it is imperative that plant-derived antimicrobial compounds be fully assessed for their feasibility for food application. This new field of research has great potential for more evaluation to meet regulatory requirements and to fully elucidate the possibility of employing antimicrobials from the extensive source of plants worldwide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Characterization of Shiga Toxigenic *Escherichia coli* O157 and Non-O157 Isolates from Ruminant Feces in Malaysia

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Shiga toxigenic *Escherichia coli* (STEC) O157 and several other serogroups of non-O157 STEC are causative agents of severe disease in humans world-wide. The present study was conducted to characterize STEC O157 and non-O157 serogroups O26, O103, O111, O121, O45, and O145 in ruminants in Malaysia. A total of 136 ruminant feces samples were collected from 6 different farms in Peninsular Malaysia. Immunomagnetic beads were used to isolate *E. coli* O157 and non-O157 serogroups, while PCR was used for the detection and subtyping of STEC isolates. STEC O157:H7 was isolated from 6 (4%) feces samples and all isolates obtained carried stx_{2c}, *eaeA*-*γ*1, and *ehxA*. Non-O157 STEC was isolated from 2 (1.5%) feces samples with one isolate carrying stx_{1a}, stx_{2a}, stx_{2c}, and *ehxA* and the other carrying stx_{1a} alone. The presence of STEC O157 and non-O157 in a small percentage of ruminants in this study together with their virulence characteristics suggests that they may have limited impact on public health.

1. Introduction

Shiga toxin producing *E. coli* (STEC), a serologically diverse group of zoonotic pathogens, have emerged as one of the most virulent groups of bacteria associated with cases of food borne disease in humans [1]. STEC can cause a spectrum of diseases ranging from mild diarrhea to severe bloody diarrhea, called hemorrhagic colitis (HC), and even life-threatening sequelae such as hemolytic uremic syndrome (HUS). Patients with HUS were often diagnosed as having thrombotic thrombocytopenic purpura (TTP), although thrombotic microangiopathy is now considered a more accurate description of the condition associated with HUS caused by STEC [2]. Production of Shiga toxin (Stx) is considered as the major virulence factor of STEC [1] which contributes to the development of HUS in humans [2]. Stx production alone is not sufficient for STEC to cause disease. Accessory virulence factors include a 34 kb chromosomal pathogenicity island called the "locus for enterocyte effacement" (LEE) carrying several virulence associated genes, such as the attaching and effacing (*eaeA*) gene, and a large plasmid (60 MDa) with an *ehxA* gene encoding an enterohemolysin. *EaeA* encodes an outer-membrane protein called intimin which enables the intimate adherence of STEC to the intestinal epithelium of the host [3]. The enterohemolysin protein is implicated in extracting iron from the blood released into the intestine [4].

The prototype STEC serotype is *E. coli* O157:H7 and its ability to cause HC and HUS in many regions and countries is well established. The pathogenic potential and public health significance of several non-O157 STEC serogroups, particularly O26, O103, O111, O121, O145, and O45 referred to as the "big 6" non-O157 STEC serogroups [5], have also been described in recent years due to their association with clinical HC and HUS in humans. In some geographical areas, such as in Europe, the disease caused by non-O157 strains is significantly more common than that caused by O157:H7 [6, 7].

Ruminants are considered an important source of both *E. coli* O157 and non-O157 with cattle being identified as the primary reservoir. Intestinal carriage of *E. coli* O157 and non-O157 in ruminants results in their fecal shedding and release into the environment. As a result, infections of *E. coli* O157 and non-O157 can be transmitted to humans via the consumption of food and water contaminated by animal feces.

Data on *E. coli* O157 and non-O157 serotypes in ruminants is limited in countries of the tropical regions including Malaysia. In addition, the data reported so far on *E. coli* O157

Table 1: Distribution of ruminant feces samples collected from farms A–F.

Farm	Location	Ruminant feces samples				Total samples
		Cattle	Buffalo	Goat	Sheep	
A	Serdang	25	—	—	—	25
B	Kluang	9	20	7	8	44
C	Sentul	9	—	—	—	9
D	Gemas	24	—	—	—	24
E	Puchong	13	—	5	—	18
F	Lumut	16	—	—	—	16

and non-O157 in ruminants from tropical countries other than Malaysia demonstrates substantial variation in their prevalence and virulence properties. In West Bengal, India, a total of fourteen STEC O157 isolates were obtained from two (2.04%) slaughtered cattle feces samples and six (7.59%) diarrhoeic calf feces samples [8]. The majority of STEC O157 isolates (85.71%) obtained from this study carried stx_2 alone. STEC O157 was obtained from 0.6% of cattle feces samples in Brazil [9], where the majority of isolates carried *ehxA* either with both stx_1 and stx_2 or with stx_2 alone. The prevalence of *E. coli* O157 was found to be 1.25% in cattle farms in central Mexico [10]. Non-O157 STEC was found in 18% of cattle feces samples in Calcutta, India [11], in which stx_1 predominated. In another study in Brazil, non-O157 STEC was isolated from 5.81% of calf feces samples [12] where stx_1 was the dominant *stx* genotype observed.

Only three studies which isolated STEC O157 from beef samples have to our knowledge been conducted in Malaysia [13–15]. Apart from a single study which reported sporadic cases of STEC O157 infection among 14% of patients presented with bloody diarrhea at a local hospital in Kuala Lumpur, Malaysia [16], there are no other published reports of sporadic cases or outbreaks of STEC O157 and non-O157 in the country. Although studies have demonstrated the presence of STEC O157 in foods of animal origin, the presence and characterization of STEC O157 or non-O157 in ruminant feces from Malaysia has not yet been determined.

The aim of the present study was to examine ruminant feces samples for the presence of STEC O157 and the "big 6" non-O157 STEC serogroups in Malaysia. The isolated strains of *E. coli* O157 and non-O157 were further characterized to determine their genetic diversity and presence of virulence factors to indicate the risk potential of these strains to public health.

2. Materials and Methods

2.1. Sample Collection and Preparation. Samples were collected from six different ruminant farms in Peninsular Malaysia (Table 1). The geographical distribution of the six farms is depicted in Figure 1. Farms A, C, and F were small dairy cattle farms, while farm E was a small dairy farm consisting of cattle and goats. Farm B was also a dairy farm but with a larger number and diversity of ruminants consisting of cattle, buffaloes, goats, and sheep. Farm D was a large beef cattle farm. A total of 136 fresh ruminant feces samples (~25 g each) from cattle, buffalo, sheep, and goat

Figure 1: Geographical distribution of farms A–F in Peninsular Malaysia from which the ruminant feces samples were collected.

were collected from the pen floors (over a period of six months) into sterile containers and were stored at 4°C on ice until processed in the lab on the same day. All fecal samples collected were divided into two 10 g samples. One of the 10 g samples was used for enrichment and the other was used for long term storage in tryptone soy broth (TSB; Merck, Darmstadt, Germany) with 25% glycerol at −70°C.

2.2. Isolation and Characterization of E. coli O157. Each fecal sample (10 g) was diluted 1/10 in buffered peptone water (BPW; Oxoid, Hampshire, UK) and homogenized for 30 s. Samples were incubated for 18 h at 37°C without agitation. Immunomagnetic separation (IMS) was performed using Dynabeads anti-*E. coli* O157 (Dynal, Oslo, Norway) according to the manufacturer's instructions. Resulting bead-bacteria complexes were spread on to sorbitol-MacConkey agar (SMAC; Oxoid, Hampshire, UK) and sorbitol-MacConkey agar containing the cefixime, tellurite supplement (CT-SMAC; Oxoid, Hampshire, UK) and incubated for 18 h

at 37°C. A total of 10 presumptive *E. coli* O157 colonies per sample were serotyped using an *E. coli* O157 Latex Test Kit (Oxoid, Hampshire, UK). All isolates agglutinating with the O157 antiserum were further characterized by polymerase chain reaction (PCR) to detect the presence of *rfbE, stx*$_1$, *stx*$_2$, *eaeA, ehxA*, and *fliC* genes using primers and reaction conditions as previously described [17].

Characterization of lineage-specific polymorphisms-6 (LSPA-6) of *E. coli* O157 isolates was performed using target amplification and capillary electrophoresis as described previously [18, 19]. An Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, California, USA) with a DS-33 matrix and GeneScan 600 LIZsize standard was used for capillary electrophoresis, while a Peak Scanner software (Version 1.0; Applied Biosystems, California, USA) was used to interpret amplicon sizes. LSPA-6 alleles were defined according to [18]. Isolates with LSPA-6 genotype 111111 or 211111 were classified as lineage I (LI) or lineage I/II (LI/II), respectively, while all other allele combinations were grouped as lineage II (LII) [18, 20].

Analysis of Shiga toxin encoding bacteriophage insertion sites (SBI) of *E. coli* O157 isolates was determined as previously described [21].

2.3. Detection, Isolation, and Characterization of Non-O157 E. coli. Samples (10 g) which were initially stored at −70°C in TSB with 25% glycerol were diluted 1/10 in BPW, homogenized for 30 s, and incubated for 18 h at 37°C without agitation. DNA was extracted from 1 mL of the enriched sample using the Nucleospin Soli DNA extraction kit (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. A multiplex PCR was used to screen enrichments for the presence of STEC virulence genes *stx*$_1$, *stx*$_2$, *eaeA*, and *ehxA* using primers and reaction conditions as described by A. W. Paton and J. C. Paton [22] with several modifications. A reaction volume of 25 μL was used with 2 μL of DNA template and final concentration of 0.25 μM of each primer, 5x Green GoTaq Flexi Buffer (Promega, Madison, USA), 200 μM of dNTP, 2 mM of $MgCl_2$, and 1 unit of GoTaq DNA polymerase (Promega, Madison, USA). The PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide (0.5 μg/mL) and visualized under UV light. Enriched samples positive for *stx* and *eaeA* by PCR were streaked on chromocult-TBX agar (Merck, Darmstadt, Germany) and coliformen agar enhanced selectivity (Merck, Darmstadt, Germany) and incubated overnight at 37°C. Following incubation, up to 50 *E. coli* colonies per sample were chosen based on colony morphology and screened individually by multiplex PCR for the presence of *stx*$_1$, *stx*$_2$, *eaeA*, and *ehxA* as described above. Colonies that were positive for *stx* and *eaeA* were then tested for the "big 6" *E. coli* non-O157 serogroups by PCR using primers and conditions described previously [17, 23].

The enriched samples were also tested for the presence of genes specific to the "big 6" *E. coli* non-O157 serogroups. Samples that tested positive by PCR for any of the target serogroups were subjected to IMS for O26, O111, O103, and O145 using Dynabeads (Dynal, Oslo, Norway) following the manufacturer's instructions. The bead-bacteria complexes formed during IMS of O26 were plated onto rhamnose MacConkey agar, while those of O111, O103, and O145 were plated onto chromocult-TBX agar and coliformen agar-enhanced selectivity and incubated overnight at 37°C. Following incubation, 10 presumptive colonies (per sample) based on colony morphology were subjected to serogroup specific PCR and those confirmed as a specific serogroup were tested by PCR for the presence of STEC virulence genes. Isolation of serogroups O45 and O121 was performed on enriched fecal samples positive for STEC virulence markers which were directly plated onto chromocult-TBX agar as described above.

2.4. Biochemical Confirmation of E. coli Isolates. All the isolates were biochemically identified as *E. coli* by citrate utilization and indole production tests [24].

2.5. Bacterial Strains. The bacterial strains used as controls in this study are listed in Table 2.

2.6. Pulsed-Field Gel Electrophoresis (PFGE). PFGE using *Xba*I was performed on all *E. coli* O157 and non-O157 isolates in a CHEF Mapper (Bio-Rad, California, USA) according to the standardized PulseNet protocol [25]. Banding patterns were analysed using BioNumerics software, version 6.5 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) following the PulseNet protocol.

2.7. Subtyping of stx and Intimin (eaeA) Genes of E. coli O157 and Non-O157. The subtypes of *stx* and *eaeA* in isolates carrying these markers were determined following previously published methods [26, 27].

2.8. Detection of Shiga Toxin Expression. Stx expression by the STEC strains was determined according to the method adapted from Shringi et al. [28] using an ELISA kit (Premier EHEC, Meridian Bioscience, Ohio, USA). Mitomycin C (Sigma Aldrich, Missouri, USA) was used at a final concentration of 0.5 μg/mL to induce Stx production. After induction, the cells were lysed using Polymixin B (Sigma Aldrich, Missouri, USA) at a final concentration of 0.5 mg/mL and incubated at 37°C for 1 h with rotary shaking (250 rpm). Polymixin B treated cultures were diluted 1 : 100 in sterile LB broth immediately followed by 1 : 2 dilution in sample diluent of the ELISA kit. Absorbance readings were obtained at wavelengths 450 nm and 630 nm using a Victor X microtiter plate reader (Perkin Elmer, Glen Waverley, Australia) and the results were displayed as the mean value of two independent biological replicates.

3. Results

3.1. Presence of STEC O157 and Virulence Factors. STEC O157 was isolated from 6 (4%) cattle feces samples, all of which were from farm A (Table 3). A total of 32 STEC O157 isolates were obtained from 6 different cattle feces samples. The isolates obtained were clustered into two different PFGE groups (at >92% similarity) with the majority of isolates (28 isolates from 5 different fecal samples) belonging to one PFGE

Table 2: Bacterial strains used in the study.

Strain ID	Serogroup	Source	Country	Virulence traits
Sakai	O157	Radish sprouts	Japan	stx_1, stx_2, *eaeA*, *ehxA*
ATCC 43895	O157	Ground beef	USA	stx_1, stx_2, *eaeA*, *ehxA*
EC543a	O157	Cattle feces	Australia	stx_1, stx_2, *eaeA*, *ehxA*
EC6a	O157	Cattle feces	Australia	stx_2, *eaeA*, *ehxA*
1 UPM[a]	O157	Bovine milk	Malaysia	stx_1, stx_2, *eaeA*, *ehxA*
2 UPM[a]	O157	Bovine milk	Malaysia	stx_1, stx_2, *eaeA*, *ehxA*
3 UPM[a]	O157	Beef	Malaysia	stx_1, stx_2, *eaeA*, *ehxA*
4 UPM[a]	O157	Beef	Malaysia	stx_1, stx_2, *eaeA*, *ehxA*
MG1655 (*E. coli* K-12)	OR:H48:K-[b]	Laboratory strain	USA	None
EC3008a[c]	O26	Cattle feces	Australia	*eaeA*
EC3009a[c]	O45	Cattle feces	Australia	None
EC2998a[c]	O103	Cattle feces	Australia	None
EC3113a[c]	O111	Cattle feces	Australia	None
EC3111a[c]	O121	Cattle feces	Australia	None

[a]Provided by Professor Son Radu at Universiti Putra Malaysia.
[b]OR = O antigen rough strain which does not produce a typeable O antigen.
[c]*E. coli* non-O157 strains used as controls in the study, provided by Lesley Duffy at CSIRO, Brisbane, Australia.

Table 3: STEC O157 and non-O157 and their virulence profiles.

STEC serogroup	Number of STEC+ samples (%)	Source	Number of isolates	Virulence factors	Lineage	SBI profile
O157:H7[a]	6 (4%)	Cattle feces	28[b]	stx_{2c}, *eaeA*-γ1, *ehxA*	II	SY2c
			4[b]	stx_{2c}, *eaeA*-γ1 *ehxA*	II	SY2c
Non-O157[a] (unknown)	2 (1.5%)	Cattle feces	1	stx_{1a}, stx_{2a}, stx_{2c}, *ehxA*	—	—
			1	stx_1	—	—

[a]Isolates of STEC O157:H7 and non-O157 were only present in samples obtained from farm A. All samples from farms B–F were negative for STEC O157:H7 and non-O157 isolates.
[b]On farm A, 28 of the STEC O157 isolates belonged to one PFGE group (at >92% similarity) and the remaining 4 isolates belonged to another PFGE group.
—: not applicable.

group and the remaining isolates (4 isolates from a single fecal sample) belonging to the other. All 32 STEC O157 isolates were positive for the virulence factors stx_2, *eaeA*, and *ehxA* and also for *fliC* specific for the H7 antigen indicating they belong to the O157:H7 genotype. All samples from farms B–F were negative for the presence STEC O157.

LSPA-6 target amplification indicated that all the STEC O157:H7 isolates collected from cattle feces samples in farm A belong to lineage II (Table 3). According to the SBI genotyping code, genotype SY2c was observed in all STEC O157:H7 isolates collected from cattle feces samples in farm A indicating the association of stx_{2c} with prophage insertion in the *sbcB* locus (Table 3).

In addition, all STEC O157:H7 isolates obtained from UPM carried the virulence markers stx_1, stx_2, *eaeA*, and *ehxA* and belonged to a single PFGE group (at >92% similarity). They were of lineage I and contained the SBI genotype WY12 indicating the association of stx_1 and stx_{2a} with prophage insertion in the *yehV* and *wrbA* loci, respectively.

3.2. Presence of Non-O157 STEC and Virulence Factors. In the initial PCR screen of the enriched samples, various combinations of virulence markers and genes for the target non-O157 serogroups were observed in all the farms except in farm F. Although samples in farm F were positive for different combinations of virulence markers, none of the samples were positive for any of the target non-O157 serogroups tested (Table 4). Overall, the combination of *stx* (either stx_1, stx_2 or both) and *eaeA* was present in 32.3% (44 samples out of 136 samples), while the gene indicating the presence of serogroup O103 seemed to be predominant (44.1% of samples) among all 136 samples.

Although the initial PCR screening of the enriched feces samples indicated a relatively high number of samples with the target genes for the virulence factors and non-O157 serogroups, only 2 samples (1.5%) yielded non-O157 STEC isolates (Table 3). Both of these were cattle feces samples collected from farm A, from which two non-O157 STEC strains (negative for any of the "big 6" non-O157 serogroups)

Table 4: Occurrence of target virulence factors and "big 6" non-O157 serogroups in the initial PCR screen of the enriched samples from each farm (A–F).

Farm	Number of samples tested	Percent positive for virulence gene combinations[a]								Percent positive for serogroups[a]					
		stx$_1$, *stx*$_2$, *eaeA*, *ehxA*	*stx*$_1$, *stx*$_2$, *ehxA*	*stx*$_1$ or *stx*$_2$, *eaeA*, *ehxA*	*stx*$_1$, *ehxA*	*stx*$_2$, *ehxA*	*eaeA*, *ehxA*	*eaeA*	*stx*$_1$ or *stx*$_2$ alone	O111	O26	O121	O145	O45	O103
A	25	6.6	2.9	0	0	1.5	0	0	0	0	0	5.9	0	3.7	5.1
B	44	13.2	2.9	0	0	0	0	0	0	0.7	9.6	13.2	0	2.2	16.2
C	9	2.9	0	0	0	0.7	2.2	0.7	0	0	2.9	0.7	1.5	3.7	5.1
D	24	1.5	4.4	1.5	0	0	0.7	0	2.9	0	8.1	0	0.7	0.7	10.3
E	18	5.9	2.9	0.7	0	0	0	0	0	3.7	4.4	0.7	0.7	8.1	7.4
F	16	0	2.2	0	1.5	0	0	0	0	0	0	0	0	0	0
Total	136	30.1	15.4	2.2	1.5	2.2	2.9	0.7	2.9	4.4	25	20.6	2.9	18.4	44.1

[a]The percentage of samples positive were calculated by dividing the number of positive samples for each category in the initial PCR screen by the total number of samples ($n = 136$) collected.

Table 5: Isolation and virulence profiles of *E. coli* O157 and "big 6" *E. coli* non-O157 serogroups lacking *stx*.

Serogroup	Farm	Source	Number of + samples	Number of isolates tested	Virulence factors	Intimin subtype
O103	A	Cattle feces	3	3	None	—
O157	B	Cattle feces	1	2	*eaeA*	NT[a]
		Sheep feces	2	3	*eaeA* (1 isolate) none (2 isolates)	NT
		Buffalo feces	3	5	*eaeA*	NT
O26	B	Buffalo feces	2	5	*eaeA*, *ehxA* (2 isolates) none (3 isolates)	*eaeA-β1*
O103	B	Buffalo feces	1	7	None	—
O26	C	Cattle feces	2	11	None	—

[a]NT = non-typable.
—: not applicable.

were isolated which belonged to two unique PFGE groups. One of the two non-O157 STEC isolates was positive for stx_1, stx_2, and *ehxA* while the other isolate was positive for stx_1 alone.

3.3. Characterization of E. coli Serogroups Lacking stx. *E. coli* of the target serogroups (O157 and the "big 6" non-O157) lacking *stx* but carrying other combinations of virulence markers were also isolated from ruminant feces samples (Table 5). These included *E. coli* of serogroups O157, O103, and O26 which were negative for any of the virulence markers, O157 which carried *eaeA* alone and O26 with *eaeA* and *ehxA*.

3.4. stx and eaeA Subtyping. All the STEC O157:H7 isolates collected from cattle feces samples in farm A were positive for stx_{2c} (Table 3), while all STEC O157:H7 isolates from UPM were positive for stx_{1a} and stx_{2a}. One of the non-O157 STEC isolates was positive for stx_{1a}, stx_{2a}, and stx_{2c}, while the other isolate was positive for stx_{1a} alone (Table 3). Overall, stx_{2c} was the more prevalent genotype among the stx_2 positive isolates.

Two different *eaeA* subtypes out of the seven *eaeA* variants ($\alpha1$, $\alpha2$, $\beta1$, $\beta2$, $\gamma1$, $\gamma2/\theta$, and ε) tested were present among the *eaeA* positive isolates of *E. coli* O157 and non-O157. The STEC O157:H7 isolates were positive for *eaeA*-$\gamma1$ (Table 3) while the two O26 isolates lacking *stx* were positive for *eaeA*-$\beta1$ (Table 5).

3.5. Shiga Toxin Production. Stx production of all the stx_{2c} positive STEC O157:H7 isolates collected from the cattle feces samples in farm A were below the level of detection. In contrast, all the stx_{1a} and stx_{2a} positive STEC O157:H7 isolates obtained from UPM produced a high amount of Stx similar to the positive control STEC O157:H7 isolates, ATCC 43895, EC543a, and EC6a. Of the two non-O157 STEC isolates, Stx production of the stx_{1a} positive non-O157 isolate was also below the level of detection. However, the stx_{1a}, stx_{2a}, and stx_{2c} positive non-O157 STEC isolate indicated a moderate amount of Stx production although lower than that observed for the UPM STEC O157:H7 isolates.

4. Discussion

In Malaysia, no studies have been conducted so far to characterize STEC O157 or non-O157 in ruminant feces. Thus, the goal of the present study was to gain insight on the virulence determinants of STEC O157 and non-O157 present in ruminant feces in Malaysia.

In this study, STEC O157 was isolated from 6 (4.4%) ruminant feces samples and non-O157 STEC was isolated from 2 (1.5%) of the ruminant feces samples. Several other authors have also reported low isolation rates (less than 10%) of STEC O157 and non-O157 in ruminant feces in tropical countries [8, 12]. However, this study was not adequate to determine the prevalence of STEC O157 and non-O157 in Malaysia and, thus, to obtain more comprehensive data on the prevalence of STEC O157 and non-O157 serogroups in Malaysia, sampling of a wider geographical area within Malaysia should be undertaken.

E. coli O157 populations have been shown to vary in their distribution among bovine and clinical sources due to their genotypic differences [29]. LSPA-6 analysis, a simple multiplex PCR assay, categorizes *E. coli* O157 strains into three different genotypes referred to as lineage I, lineage I/II, and lineage II. Isolates of lineage I and I/II are mostly associated with human clinical sources while lineage II isolates are mostly associated with bovine sources [18]. In this study, all the STEC O157:H7 isolates belonged to lineage II in contrast to STEC O157 isolates from countries such as Australia and USA where lineage I/II and lineage I predominates [30]. Interestingly, all the STEC O157:H7 isolates from UPM were of lineage I indicating the presence of STEC O157 isolates of both lineage I and II in bovine sources in Malaysia. STEC O157 isolates of lineage II are shown to be less virulent and possibly impaired in their transmissibility to humans compared to lineage I or I/II [31]. The presence of STEC O157 isolates of lineage II in ruminants in Malaysia from this study suggests that these isolates could have less pathogenic potential in humans.

Pathogenic potential of STEC isolates has also been shown to be associated with the presence of particular *stx* genotypes. *E. coli* isolates carrying stx_1 or stx_{2c} are associated with low virulence potential compared to those which carry

stx_2 (stx_{2a}) [32]. In this study, all the STEC O157:H7 isolates obtained carried stx_{2c} indicating low virulence potential in humans compared to the STEC O157:H7 isolates from UPM with stx_{1a} and stx_{2a}. One of the two non-O157 STEC isolates of unknown serogroup with stx_{1a}, stx_{2a}, and stx_{2c} indicated a high pathogenic potential compared to the other isolate with stx_{1a} alone.

Not all *E. coli* isolates carrying *stx* produce Stx [33]. This was true for all stx_{2c} positive STEC O157:H7 isolates and one of the non-O157 isolates positive for stx_{1a} obtained in this study. In contrast, the UPM STEC O157 isolates produced Stx. Although the exact reasons for the discrepancy observed in Stx production of *stx* positive *E. coli* isolates from this study is not fully understood, previous studies have also identified *E. coli* isolates positive for *stx* but negative for Stx production [33, 34]. In fact, the study by Koitabashi et al. [34] suggested that stx_2 positive *E. coli* O157 strains that produce little or no Stx2 may be widely distributed in the Asian environment.

Particular *stx* genotypes of STEC O157 have been shown to be associated both with particular SBI genotypes and with their relative frequency of isolation from clinical and bovine sources [21]. Clinical isolates are generally characterized by the carrying of stx_2 and stx_2-associated bacteriophage sequences adjacent to either *wrbA* or *argW* (SBI genotypes: WY12, AY2, ASY2, ASY22c), while bovine isolates are characterized by carrying of stx_{2c} and stx_{2c}-associated bacteriophage sequences adjacent to *sbcB* (SBI genotypes: SY2c, SY12c, and ASY12c). In agreement with these observations, the STEC O157 isolates obtained from cattle feces from this study carried stx_{2c} with an occupied *sbcB* locus (SY2c). However, the STEC O157 from UPM which were collected from bovine sources carried stx_2 and an occupied *wrbA* locus indicating characteristics of clinical isolates.

All the STEC O157:H7 isolates in this study and the STEC O157:H7 isolates from UPM carried *eaeA*-γ1 as reported for *eaeA* positive *E. coli* O157 in previous studies [27, 35]. None of the *eaeA* positive non-STEC O157 could be subtyped using the primers for *eaeA* subtypes α1, α2, β1, β2, γ1, γ2/θ, and ε. It is possible that these isolates belonged to other intimin subtypes such as δ/κ, ζ, η, ι, λ, μ, and ν which were not tested for in this study. The two *eaeA* positive *E. coli* O26 isolates carried *eaeA*-β1 similar to several other *E. coli* O26 isolates previously associated with human STEC strains that cause HUS [27].

5. Conclusions

Despite the use of specific and sensitive methods of enrichment and IMS followed in this study to isolate STEC O157 and non-O157, it appears that the presence of both STEC O157 and non-O157 in ruminant feces was low (4% and 1.5%, resp.). The stx_{2c} carrying STEC O157:H7 isolates of lineage II from this study suggests that these bacteria potentially represent a less pathogenic clone of STEC O157 in Malaysia. This together with the presence of STEC O157 and non-O157 in a small percentage of ruminants in this study could contribute to the reasons for the lack of reported sporadic cases and outbreaks caused by STEC O157 in Malaysia. Similar to STEC O157, the low percentage of non-O157 STEC isolates observed together with their low pathogenic potential indicated by the lack of *eaeA* and moderate to no Stx production suggests a low probability of causing disease in humans.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Impact of Moderate Heat, Carvacrol, and Thymol Treatments on the Viability, Injury, and Stress Response of *Listeria monocytogenes*

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The microbial safety and stability of minimally processed foods are based on the application of combined preservative factors. Since microorganisms are able to develop adaptive networks to survive under conditions of stress, food safety may be affected, and therefore understanding of stress adaptive mechanisms plays a key role in designing safe food processing conditions. In the present study, the viability and the sublethal injury of *Listeria monocytogenes* exposed to moderate heat (55°C) and/or essential oil compounds (carvacrol and thymol, 0.3 mM) treatments were studied. Synergistic effects were obtained when combining mild heat (55°C) with one or both essential oil compounds, leading to inactivation kinetics values three to four times lower than when using heat alone. All the treatments applied caused some injury in the population. The injury levels ranged from around 20% of the surviving population under the mildest conditions to more than 99.99% under the most stringent conditions. Protein extracts of cells exposed to these treatments were analysed by two-dimensional gel electrophoresis. The results obtained revealed that stressed cells exhibited differential protein expression to control cells. The proteins upregulated under these stressing conditions were implicated, among other functions, in stress response, metabolism, and protein refolding.

1. Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen microorganism. This microorganism causes a disease called listeriosis, which has been associated with outbreaks by ingestion of milk, cheese, vegetables, salads, and meat [1, 2]. The pervasiveness of this microorganism is due, in part, to its ability to tolerate extreme environment conditions (high salt concentration, wide range of pH and temperature, and low water availability) [3].

Food manufactures and consumers demand additive-free, fresher, and full tasting food products while maintaining high standards of microbiological safety. The use of natural antimicrobial systems for preservation of foods could accomplish this demand. Although essential oil components are used as flavourings in the food industry, nowadays they represent a highly interesting source of natural antimicrobials for food preservation due to their antimicrobial and antioxidative activity [4]. Carvacrol and thymol are natural phenolic compounds present in the essential oil fraction of *Origanum* and *Thymus* plants [5, 6] and have long been used in foods as flavour enhancers. Both compounds of essential oils (carvacrol and thymol) have been shown to exhibit antibacterial and antifungal activity including food pathogens [5–10]. A synergistic effect of nisin, carvacrol, and thymol [9, 11] or thymol and cymene [7, 12] against vegetative cells of *B. cereus* has been observed. When a mild thermal treatment was applied prior to the growth in presence of antimicrobials, the sensitivity to antimicrobials was increased [8, 11]. Likewise, a synergistic effect of carvacrol and cymene [13] or nisin

and carvacrol [14] to control growth and viability of *Listeria monocytogenes* has also been shown. Again, heat was able to increase *L. monocytogenes* sensitivity to antimicrobials [14]. The antimicrobial property of carvacrol and thymol has been attributed to the considerable effects on the structural and functional properties of cytoplasmatic membrane [15]. Cell membrane alterations caused by these compounds are able to induce sublethal injury.

As sublethal injury is supposed to be related to the higher sensitivity of survivors to stress conditions after treatment, the success of a combined treatment should be correlated with the degree of sublethal injury caused by the hurdles in the bacterial population [16]. Moreover, under suitable conditions, sublethal injured cells might be repaired, which is a very important aspect to be taken into account regarding food safety. A few cells being capable of repairing damage after moderate heat and essential oil compounds treatment could result in infective concentrations.

The microbial safety and stability of most minimally processed foods are based on application of combined preservative factors of which (mild) heating is the most common preservation technique in use these days. Bacteria have evolved adaptive networks to face the challenges of changing environments and to survive under conditions of stress [17]. When bacterial cells, grown at an optimal temperature, are shifted to a higher temperature a heat-shock response develops and heat resistance and the synthesis of a set of heat-shock proteins (HSPs) are induced. Bacterial heat resistance is affected by a wide variety of genetic, physiological, and environmental factors. When microorganisms develop resistance to commonly used preservation methods, food quality and safety may be affected, and therefore understanding of stress adaptive mechanisms plays a key role in designing safe food processing conditions [18].

So far, the combined effect of carvacrol, thymol, and mild heat treatments on the viability, sublethal injury, and the protein expression profile of *L. monocytogenes* cells is not well known. Therefore, in the present study, the viability and the sublethal injury of cells of *L. monocytogenes* CECT 4031 exposed to moderate heat and/or essential oils compounds (carvacrol and thymol) treatments were studied. Then, protein extracts of cells exposed to these treatments were analysed by two-dimensional gel electrophoresis (2D-electrophoresis) and 20 proteins were identified and their roles were discussed.

2. Materials and Methods

2.1. Bacterial Strains and Preparation of Inoculum. *L. monocytogenes* CECT 4031, provided by the Spanish Type Culture Collection (CECT, Valencia, Spain), was used in the present study. Cells were grown overnight at 37°C in Tryptic Soy Broth (TSB; Scharlau, Barcelona, Spain) containing 0.6 g/100 g yeast extract (TSBYE; Scharlau). Then, *L. monocytogenes* was inoculated into fresh medium (TSBYE) and incubated at 37°C with shaking at 140 rpm until the stationary phase was reached.

2.2. Chemicals. Carvacrol (Fluka Chemie AG, Buchs, Switzerland) and thymol (Sigma Aldrich Chemie, Steinheim, Germany) stock solutions were held in 95% ethanol at 4°C.

2.3. Effect of Mild Heat Treatment and/or Essential Oils Compounds (Carvacrol and Thymol) on L. monocytogenes Cells Viability. Cultures in stationary phase, grown at 37°C, were harvested and concentrated by centrifugation (3500 ×g, 10 min, at 4°C) and resuspended in TSBYE. Treatments with only essential oil compounds were carried out through exposures of *L. monocytogenes* to carvacrol alone (0.3 mM), thymol alone (0.3 mM), or both compounds together (0.3 mM of each one) in 5 mL TSBYE medium (initial concentration of *L. monocytogenes* 10^{10} CFU/mL) at 25°C during 30 min in a water bath. These concentrations of antimicrobials did not modify the sensorial properties and only when combined led to slight changes in odour. Control experiments with the same concentration of ethanol (without essential oils) showed a stable viable count over 30 min of incubation in all cases (data not shown). For mild heat treatments, preheated 5 mL TSBYE medium (initial concentration of *L. monocytogenes* 10^{10} CFU/mL) kept at 55°C in a water bath was used. Mild heat treatments alone (55°C) and combined with essential oil compounds (55°C and carvacrol 0.3 mM; 55°C and thymol 0.3 mM; 55°C and carvacrol 0.3 mM and thymol 0.3 mM) during 30 min were carried out. Viable counts were analysed after 0, 5, 10, 15, 20, and 30 min of exposure. For all treatments three independent experiments were performed and samples (whose tenfold serial dilutions were prepared in buffered peptone water (Scharlau)) were plated, in duplicate, by the pour plate method on Tryptic Soy Agar (TSA, Scharlau) containing 0.6 g/100 g yeast extract (TSAYE). The plates were incubated for 24–48 h at 37°C. Survival curves were plotted as the logarithm of CFU/mL versus exposure time.

2.4. Determination of Degree Injury: Enumeration of Viable and Injured Cells. To determine the loss of viability caused by a treatment, untreated and treated cell suspensions were serially diluted and plated on the surface of an appropriate count medium. TSAYE was used as nonselective agar medium in the enumeration of viable *L. monocytogenes*. Sublethal injury of *L. monocytogenes* exposed to mild heat and/or essential oils compounds treatments was assessed by the difference between the counts on the nonselective agar medium (TSAYE) and the selective agar medium, TSAYE, supplemented with NaCl 5% (wt/vol) (Panreac, Spain) (TSAYE-SC). 5% was the maximum noninhibitory sodium chloride concentration for native cells previously determined for *L. monocytogenes* [19]. The plates were incubated for 24–48 h at 37°C. The data were presented as means of at least three independent experiments. Percent of injury was calculated for each tripled sample using the following equation [20]:

$$\%\ \text{injury} = 100 \times \frac{\text{cfu}_{\text{TSAYE}} - \text{cfu}_{\text{TSAYE-SC}}}{\text{cfu}_{\text{TSAYE}}}. \quad (1)$$

2.5. Modelling of Survival Curves. Survival curves of *L. monocytogenes* after exposure to treatments (mild heat and/or essential oils compounds) were drawn by plotting the log of microorganisms against exposure time. These curves were fitted using the cumulative function of the Weibull distribution as proposed by Mafart et al. [21] to describe the inactivation of microorganisms in terms of decimal logarithms:

$$\log_{10} N_t = \log_{10} N_0 - \left(\frac{t}{\delta}\right)^p, \quad (2)$$

where t is the treatment time, N_t and N_0 are the population densities at time t and time zero, respectively, δ is the scale parameter (time to inactivate the first logarithmic cycle of microbial population), and p is the shape parameter, which describes the behavior of the population: if $p < 1$ an upward concavity is happening, if $p > 1$ a downward concavity will be observed, and if $p = 1$ the survival curve is linear.

Since all survival curves had the same tail-shape, a single p value for all of them was used as proposed by Couvert et al. [22].

Data were analyzed using Statgraphics Plus 5.1 software (Statistical Graphics Corp., Rockville, MD). One-way analysis of variance (ANOVA) for the parameters derived from the survival experiments was used to establish significant differences between the different treatments.

2.6. Effect of Mild Heat Treatment and/or Essential Oils Compounds (Carvacrol and Thymol) on L. monocytogenes Protein Expression

2.6.1. Differential Proteome Analysis of L. monocytogenes Cells. Total cellular protein extractions were performed as described by Wouters et al. [23]. For each sample, 10 mL of stationary phase cultures of *L. monocytogenes* STCC4031 (control), exposed to mild heat alone (55°C) and combined with essential oil compounds (55°C and carvacrol 0.3 mM and thymol 0.3 mM) during 30 min, was centrifuged at 4,000 rpm during 10 minutes. The pellets were resuspended in 1 mL of lysis buffer (urea 8 M, CHAPS 2% (w/v), protease inhibitor (Roche, Germany), and DTT 5 mM). Consecutively, cells were disrupted by bead beating with a MiniBead Beater cell homogenizer (Biospec Products, Bartlesville, OK) and zirconium beads (0.1 mm diameter; Biospec Products) six times for 1 min (with cooling on ice between treatments). The protein concentration in cell-free extracts was determined using the kit RC-DC protein assay (Bio-Rad). Samples of homogenate were stored at −80°C.

2.6.2. Two-Dimensional Gel Electrophoresis. 2D-electrophoresis was performed, as described [24, 25], using gradient Immobiline Dry Strips pH 4–7 (7 cm; Amersham Biosciences) for the first dimension (IEF). Proteins samples (20 μg) were carried to a 125 μL dehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer (pH 4), 50 mM DTT, and bromophenol blue traces) and loaded onto the IPG strip. Isoelectric focusing was performed in IPGPhor-I (Amersham Biosciences) to reach 6500 Vh in total. After the separation, the first dimension strips were equilibrated twice with equilibration buffer (6 M urea, 50 mM Tris–HCl pH 8.8, 20% glycerol, and 2% SDS) in the presence of 1% DTT in the first equilibration and 4% iodoacetamide in the second one. SDS-PAGE in the second dimension was performed in 12% polyacrylamide home-casted gels. Gels were run in a Mini-Protean System (Bio-Rad) until the tracking dye reached the bottom of the gel.

2.6.3. Gel Staining and Image Analysis. After electrophoresis, the gels were stained with MALDI TOF compatible silver nitrate using a Plus One Silver Staining Kit (Amersham Biosciences), as described by Shevchenko et al. [26]. Gels were scanned with an ImageScanner II Desktop (Amersham Biosciences) and the image analysis of the gels was performed using PD Quest 8.0.1 software (Bio-Rad). Three gels were produced from independent cultures of each condition and representative image gels are shown. Induction factors for each induced protein were calculated as the ratio between the normalized value in "treated" gel and the normalized value in "control" gel. Protein spots displaying ≥2-fold change in abundance were chosen for analysis by mass spectrometry.

2.6.4. Protein Identification. Spots were manually excised from stained gels and sent for the digestion, the analysis by MS/MS, and the database searching to the Proteomics Lab of the Centro Nacional de Biotecnología (CNB-CSIC, Madrid, Spain). The digestion protocol used was based on Shevchenko et al. [26]. The MS/MS analysis was carried out in a MALDI-TOF/TOF (4800 Plus MALDI-TOF/TOF Analyzer, Applied Biosystems). Measured tryptic peptide mass values were transferred using the MS Bio Tools (Bruker) for searching in the National Center for Biotechnology Information (NCBI) nonredundant database using Mascot software (http://www.matrixscience.com/; Matrix Science, London, UK) as search engine. The confidence interval for protein identification was set to ≥95% ($p < 0.05$) and only peptides with a minimum ion score of 79 were considered correctly identified.

3. Results and Discussion

3.1. Effect of Mild Heat Treatment and/or Essential Oil Compounds (Carvacrol and Thymol) on L. monocytogenes Cells Viability. L. monocytogenes cells were sensitive to all treatments applied, either heat or antimicrobials, individually or combined (Table 1). All survival curves showed a tail-shape, so the use of classical lineal models, such as the Bigelow model, was not recommended, and the nonlinear Weibull model was used instead in order to fit the survival data. Since all the survival curves showed approximately the same shape, a single p value ($p = 0.41$) was used to fit all the survival curves, as proposed by Couvert et al. [22].

Both thymol and carvacrol inactivated about one log cycle of the initial population of *L. monocytogenes* cells within the first 20 min of exposition, showing δ values of 15.58 and

Table 1: δ values (min) of *Listeria monocytogenes* CECT 4031 ($p = 0.41$) obtained in TSBYE at 55°C and/or in presence of 0.3 mM thymol and/or 0.3 mM carvacrol and recovered in TSAYE or TSAYE-SC.

	Recovery in TSAYE			Recovery in TSAYE-SC		
	δ value (min)	RMSE	r^2	δ value (min)	RMSE	r^2
Thymol	15.58	0.226	0.893	11.02	0.259	0.901
Carvacrol	12.79	0.249	0.898	11.07	0.252	0.904
Thymol + carvacrol	8.74	0.334	0.878	3.76	0.393	0.907
55°C	0.80	0.655	0.926	0.43	0.944	0.911
55°C + thymol	0.25	0.276	0.993	0.16	0.447	0.988
55°C + carvacrol	0.25	0.319	0.990	0.14	0.419	0.989
55°C + thymol + carvacrol	0.18	0.332	0.992	0.10	0.813	0.966

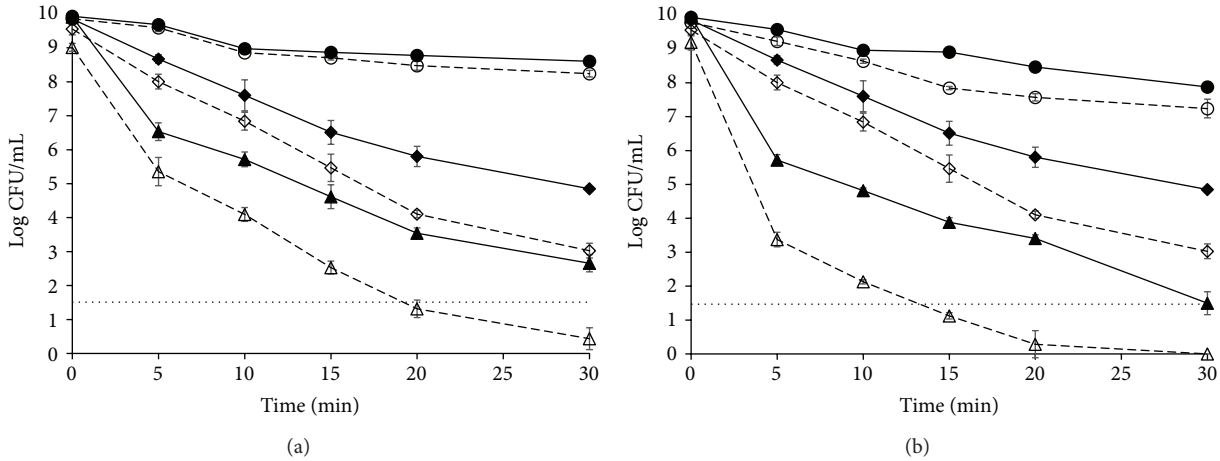

Figure 1: Survival curves of *Listeria monocytogenes* CECT 4031 obtained in TSBYE and recovered in TSAYE (continuous lines) or TSAYE-SC (dashed lines). (a) In presence of 0.3 mM thymol (●) and at 55°C (◆) and at 55°C and in presence of 0.3 mM thymol (▲). (b) In presence of 0.3 mM thymol + 0.3 mM carvacrol (●) and at 55°C (◆) and at 55°C and in presence of 0.3 mM thymol + 0.3 mM carvacrol (▲). Horizontal dotted lines show the counting technique detection limit.

12.79 min, respectively, when these antimicrobials were added to the treatment medium (Table 1). Figure 1(a) shows, as an example, the survival curve of *L. monocytogenes* exposed to 0.3 mM thymol.

When both antimicrobials were added together, approximately additive results were reached, since one log cycle was inactivated within the first 10 min of exposition and a δ value of 8.74 min was obtained (Table 1 and Figure 1(b)).

Mild heat (55°C) was much more effective in inactivating *L. monocytogenes* cells, since a δ value of 0.80 min was obtained and 5 log cycles of inactivation were reached after 30 min of treatment (Figure 1 and Table 1).

Combinations of heat with either thymol or carvacrol led again to synergistic results with δ values of 0.25 min in both cases (Table 1). Also the combination of all heat and thymol and carvacrol led to synergistic results with a δ value of 0.18 min and more than 8 log cycles' inactivation within 30 min (Figure 1(b) and Table 1).

When antimicrobials and heat are combined, synergistic results are usually obtained [27–30] with heat resistance kinetics parameters being reduced down to three times [31–33]. In our case, δ values were reduced from 0.80 min when a thermal treatment at 55°C was applied to 0.25 min when either thymol or carvacrol was added to the heating medium, which is within the average reductions. When both antimicrobials were added together to the heating medium, further reductions were obtained (0.18 min, i.e., more than four times).

3.2. Effect of Mild Heat Treatment and/or Essential Oils Compounds (Carvacrol and Thymol) on the Level of Injury. All the treatments applied caused some injury in the population of *L. monocytogenes* cells, as assessed by growth of the survivors in presence of NaCl (Figure 1 and Table 2). Injury levels ranged from very low when only thymol or carvacrol was applied for short exposition times (around 20% of the population was injured after 5 min exposition to thymol, Figure 1(a)) to more than 99% of the population where more stringent conditions were applied (Table 2). Heat caused more injury

Table 2: Percentage of injury in *Listeria monocytogenes* CECT 4031 cells after different exposure times to mild heat (55°C) applied alone or in presence of 0.3 mM thymol and/or 0.3 mM carvacrol.

Time (min)	55°C	55°C + thymol	55°C + carvacrol	55°C + thymol + carvacrol
5	76.27 ± 11.60^{aA}	92.76 ± 3.25^{aB}	98.19 ± 0.59^{aC}	99.53 ± 0.14^{aD}
10	79.78 ± 12.21^{aA}	97.25 ± 1.58^{bB}	99.16 ± 0.72^{abBC}	99.79 ± 0.06^{bC}
15	90.74 ± 2.43^{aA}	99.12 ± 0.36^{bB}	99.44 ± 0.48^{bBC}	99.82 ± 0.05^{bC}
20	97.33 ± 2.58^{bA}	99.24 ± 0.56^{bA}	99.75 ± 0.25^{bA}	99.89 ± 0.12^{bcA}
30	98.39 ± 0.69^{bA}	99.04 ± 1.02^{bAB}	99.87 ± 0.20^{bB}	$\geq 99.99^{cB}$

[a–c]The same lowercase letters indicate that there are no significant differences in columns.
[A–D]The same capital letters indicate that there are no significant differences in rows.

than antimicrobials when applied individually (Figure 1), since only 5 min at 55°C was sufficient to cause injury in more than 75% of the population (Table 2). Injured population increased as the thermal treatment proceeded. When heat and antimicrobials were combined, injured population was higher than 90% from the first moments of the treatment.

The synergistic effects of combined heat and antimicrobials have been explained in terms of inactivation of heat-injured cells when the antimicrobials are present in the heating medium [33]; that is, heat causes injuries to different cell structures but is not able to inactivate these injured cells when applied alone. However, when combined, antimicrobials would help to inactivate these injured cells. In our case, the synergistic effects of combining heat and antimicrobials could also be explained as a consequence of the inactivation of heat-injured cells by antimicrobials, since the inactivation curve of *L. monocytogenes* exposed to a mild thermal treatment and recovered in presence of NaCl is very close to that of cells exposed to a combined heat-thymol treatment and plate counts even overlap after 30 minutes of exposure (Figure 1(a)). Similar results were obtained when using carvacrol instead of thymol.

However, in our case, when heat was combined with antimicrobials additional injured population was shown, as evidenced by recovery in presence of NaCl of the survivors of this combined treatments (Table 2 and Figure 1). Hence, even when antimicrobials could help to inactivate the population injured by heat, a new injured population appeared after the combined treatment.

3.3. Effect of Mild Heat Treatment and/or Essential Oils Compounds (Carvacrol and Thymol) on L. monocytogenes Protein Expression. To gain an overview of the proteins induced upon mild heat treatment alone (55°C) and combined with essential oils compounds (55°C and carvacrol 0.3 mM and thymol 0.3 mM), 2D-electrophoresis was used. On gels containing cell-free extracts of control and treated/stressed *L. monocytogenes* cells, a total of approximately 200 spots could be detected (Figure 2). Interestingly, comparative proteomic analysis between treated and untreated *L. monocytogenes* cells showed that 54 spots exhibited different induction levels of twofold or more at least in one of the treatments applied (Table 3 and Figure 2). 38 proteins were upregulated and 16 downregulated twofold or more at least in one of the stresses applied (Table 3). The same 38 proteins were upregulated when any of the stress treatments were applied but showing different induction levels. The same happened with the 16 downregulated proteins. Surprisingly, although in our study the combined treatment (heat with both antimicrobial compounds) presented a higher antimicrobial effect on the viability of *L. monocytogenes* than heat alone, the combined treatment did not show, in general, a stronger protein response. Neither mild heat treatment alone nor mild heat treatment combined with essential oil compounds showed a specific trend in protein induction levels. Among the overexpressed protein spots, those upregulated more than twofold, after exposition either to 55°C or to 55°C and carvacrol 0.3 mM and thymol 0.3 mM, were excised from the gels and subjected to MALDI-TOF MS/MS analysis for the protein identification and determination of their biological functions. 20 proteins were successfully identified out of the whole set of overexpressed protein spots (Table 4). Spots 18, 20, 28, 32, 34, 35, 37, 38, 41, 44, and 45 were identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 3-bisphosphoglycerate-independent phosphoglycerate mutase, lactate dehydrogenase, triosephosphate isomerase, rod shape-determining protein MreB, PTS mannose transporter subunit IIAB, cysteine synthase, ATP-dependent Clp protease proteolytic subunit, transcription elongation factor GreA, hypothetical protein lmo2511, and 50 ribosomal protein L10, respectively. Spot 24 yielded two different proteins that were identified as translation elongation factor Ts and molecular chaperone DnaK and spot 47 yielded another two proteins identified as hypothetical protein lmo1580 and universal stress protein. Likewise, spot 51 showed that it contains two different proteins: regulatory protein SpoVG and 50S ribosomal protein L7/L12. Finally, spot 53 contained three different proteins: cochaperonin GroES, major cold-shock protein homolog CspB, and 50S ribosomal protein L12. According to biological functions, the 20 identified proteins belong to different categories including mainly stress response (44, 47a, 47b, and 53b), metabolic processes and their regulation (18, 20, 28, 32, 35, and 37), protein synthesis (24a, 41, 45, 51c, and 53c), protein folding (24b, 53a), and protease activity (38) (Table 4).

The results obtained in the current study revealed that *L. monocytogenes* CECT 4031 control cells exhibited differential

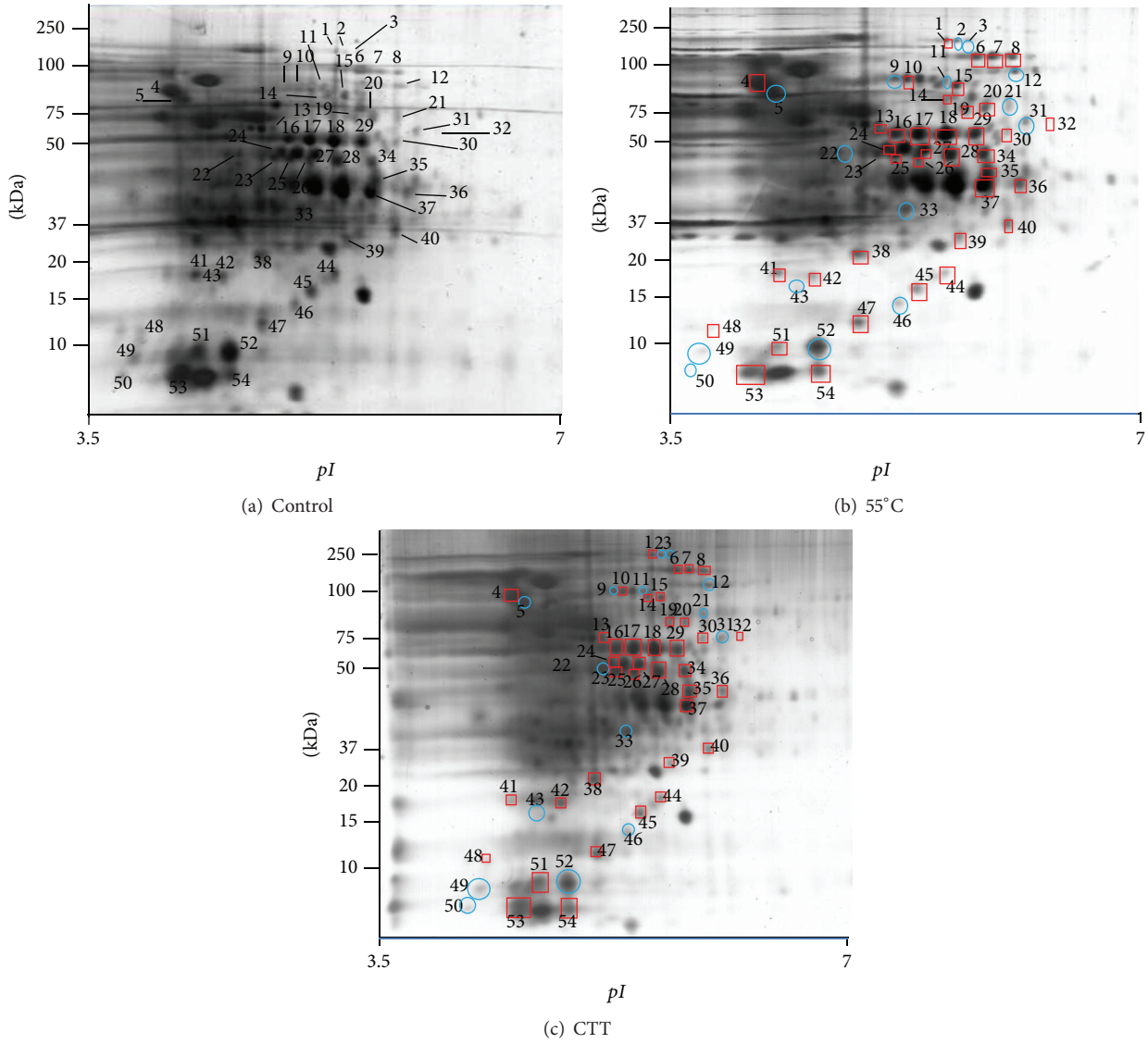

Figure 2: 2D-electrophoresis of extracts of stationary phase *Listeria monocytogenes* CECT 4031 grown in TSAYE at 37°C: (a) resuspended in TSAYE; (b) exposed to 55°C; (c) exposed to 55°C in presence of 0.3 mM thymol + 0.3 mM carvacrol, for 30 minutes. Induced proteins (squares) and repressed proteins (circles) are numerated (see also Tables 3 and 4).

protein expression to stressed cells. The proteins upregulated under these stressing conditions are implicated, among other functions, in stress response, metabolism, and protein refolding.

In bacterial cells heat causes damage to macromolecular cell components such as proteins and DNA. Carvacrol and thymol show the same mechanism of action due to their similar chemical structure. Both EOs seem to increase cytoplasmic membrane permeability [15] causing the destruction of the lipid bilayer in Gram-negative bacteria. As a consequence, there is an efflux of ions and ATP causing proton motive force dissipation and eventually cell death. To cope with these adverse conditions, response to heat stress involves protein chaperones, which assist in folding and assembly of heat damaged proteins [34] and proteases.

In our study, the treatment applied induced the overexpression in *L. monocytogenes* of the chaperone DnaK (spot 24b, Table 4), the cochaperonin GroES (spot 53a, Table 4), and ATP-dependent Clp protease subunit (spot 38, Table 4). Chaperone DnaK was also overexpressed when *L. monocytogenes* was exposed to other antimicrobial substances, such as nisin [35] or enterocin AS-48 [36]. These chaperones have been reported to be required in *L. monocytogenes* for tolerance to environmental stresses [37] and in the autolytic proteome [38]. Others studies also showed that some chaperones and proteases are heat-shock proteins (HSPs) expressed

Table 3: Induction factor[a] of proteins of *Listeria monocytogenes* CECT 4031 exposed to mild heat treatment alone (55°C) and combined with essential oils compounds (55°C and carvacrol 0.3 mM and thymol 0.3 mM) for 30 minutes.

Spot number	55°C	55°C + EOs
1	2.0	2.1
2	−4.3	−14.1
3	−4.9	−4.8
4	3.2	2.4
5	−2.1	−3.9
6	3.1	2.2
7	2.7	2.9
8	2.5	2.8
9	−1.7	−2.5
10	2.1	1.6
11	−1.5	−2.1
12	−1.8	−2.0
13	8.4	6.4
14	2.0	3.3
15	1.4	2.0
16	18.5	12.7
17	11.2	9.2
18	4.5	4.3
19	2.0	1.9
20	3.5	2.1
21	−14.7	−8.8
22	−3.3	0.0
23	0.0	−12.3
24	4.2	3.9
25	2.0	2.2
26	5.1	2.0
27	2.0	2.1
28	2.0	2.2
29	6.0	5.0
30	9.1	5.3
31	−1.6	−2.7
32	2.2	2.4
33	−7.7	−3.4
34	8.5	5.0
35	9.1	5.3
36	2.1	1.6
37	2.7	1.7
38	7.8	10.8
39	2.6	2.0
40	2.0	2.6
41	1.2	2.1
42	3.3	7.6
43	−2.0	−2.2
44	2.3	2.0
45	2.0	3.3
46	−2.0	−2.0
47	18.5	12.7
48	10.2	11.2
49	−9.6	−1.6
50	−2.5	−1.6
51	2.3	2.5
52	−13.5	−7.6
53	2.0	3.1
54	2.5	2.0

[a]Normalized value in treated gel/normalized value in control gel.

in response of *Bacillus cereus* and *Bacillus weihenstephanensis* to even moderated temperatures or other cellular stresses [39, 40].

Cell metabolism is essential for energy generation, DNA replication, and cell division. Overexpression of proteins involved in energy metabolism could be an attempt to compensate for partially impaired energy generation caused by stressing treatments interacting with the bacterial cytoplasmic membrane. In fact, our results showed that stress treatments induced the expression in *L. monocytogenes* of five proteins involved in metabolic processes, between two- and fourfold. Other authors found that proteins like glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cysteine synthase were induced in the autolytic proteome of *L. monocytogenes* [38] and exposure to nisin [35].

In our study, an overproduction of proteins of stress response, like the major cold-shock-protein CspB, was observed when treatments were applied. The same protein was reported to have been induced in *B. cereus* when exposed to stresses as heat and ethanol [40]. Interestingly, the MreB rod shape-determining protein and the regulatory protein SpoVG were also induced in our study and likewise were found in *B. cereus* exposed to moderate heat or ethanol [40] and in *L. monocytogenes* isolated from smoked mussels [41].

The phenotypic and genotypic robustness of *L. monocytogenes* is of particular concern to the food industry as it has a variety of encoded proteins implicated in survival mechanisms to withstand environmental stressors such as heat, cold, salt, acidic pH, or antimicrobials [42, 43]. In addition, some of these stress responses can result in enhanced microbial survival, enhanced thermotolerance, and even cross protection against multiple stresses [39, 40, 44].

4. Conclusions

Our results show that a combined treatment of moderate heat with antimicrobials (carvacrol and/or thymol) brings synergistic effects. Both heat and antimicrobials cause some degree of injury in the cells surviving moderate individual or combined treatments. Stress response in these injured cells involves overexpression of proteins implicated, among other

Table 4: Identification of proteins upregulated in *Listeria monocytogenes* CECT 4031 exposed to mild heat treatment alone (55°C) and combined with essential oils compounds (55°C and carvacrol 0.3 mM and thymol 0.3 mM) for 30 minutes.

Spot number	Protein identity	Accession number	Mascot score	Biological function
18	Glyceraldehyde-3-phosphate dehydrogenase	gi/16804497	728	Metabolic processes
20	3-Bisphosphoglycerate-independent phosphoglycerate mutase	gi/441475375	141	Metabolic processes
24a	Translation elongation factor Ts	gi/47014632	262	Protein synthesis
24b	Molecular chaperone DnaK	gi/16803513	105	Protein folding
28	Lactate dehydrogenase	gi/185497273	128	Metabolic processes
32	Triosephosphate isomerase	gi/16804495	601	Metabolic processes
34	Rod shape-determining protein MreB	gi/16803588	93	Determination of bacterial cytoskeleton
35	PTS mannose transporter subunit IIAB	gi/16802144	117	Regulation of metabolic and transcriptional processes
37	Cysteine synthase	gi/16802269	191	Metabolic processes
38	ATP-dependent Clp protease proteolytic subunit	gi/16804506	187	Protease activity
41	Transcription elongation factor GreA	gi/735685227	198	Protein synthesis
44	Hypothetical protein lmo2511	gi/16804549	345	Stress response
45	50 ribosomal protein L10	gi/685938168	105	Protein synthesis
47a	Hypothetical protein lmo1580	gi/16803620	204	Stress response
47b	Universal stress protein	gi/46907811	204	Stress response
51a	Regulatory protein SpoVG	gi/16802242	180	Cell division
51b	50S ribosomal protein L7/L12	gi/16802297	110	Protein synthesis
53a	Cochaperonin GroES	gi/16804108	116	Protein folding
53b	Major cold-shock protein homolog CspB	gi/1864167	105	Stress response
53c	50S ribosomal protein L12	gi/786164	58	Protein synthesis

functions, in stress response, metabolism, and protein refolding. This stress response could result in enhanced survival, compromising the safety of foods preserved by combined processes.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bayesian Estimation of the True Prevalence and of the Diagnostic Test Sensitivity and Specificity of Enteropathogenic *Yersinia* in Finnish Pig Serum Samples

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Bayesian analysis was used to estimate the pig's and herd's true prevalence of enteropathogenic *Yersinia* in serum samples collected from Finnish pig farms. The sensitivity and specificity of the diagnostic test were also estimated for the commercially available ELISA which is used for antibody detection against enteropathogenic *Yersinia*. The Bayesian analysis was performed in two steps; the first step estimated the prior true prevalence of enteropathogenic *Yersinia* with data obtained from a systematic review of the literature. In the second step, data of the apparent prevalence (cross-sectional study data), prior true prevalence (first step), and estimated sensitivity and specificity of the diagnostic methods were used for building the Bayesian model. The true prevalence of *Yersinia* in slaughter-age pigs was 67.5% (95% PI 63.2–70.9). The true prevalence of *Yersinia* in sows was 74.0% (95% PI 57.3–82.4). The estimates of sensitivity and specificity values of the ELISA were 79.5% and 96.9%.

1. Introduction

Yersiniosis is a foodborne disease in humans, which is caused by *Yersinia enterocolitica* and to a lesser extent by *Yersinia pseudotuberculosis*, and it is the third most reported zoonotic disease in the EU [1]. *Y. enterocolitica* infections have been associated with the consumption of pork products [2–4]. Often healthy pigs are asymptomatic carriers of *Y. enterocolitica*; and they are a major reservoir for human pathogenic strains [3, 5, 6].

Diagnostic tests are used for prevalence surveys. Ideally, true prevalence should be estimated from apparent prevalence adjusting for the diagnostic test sensitivity and specificity [7]. It is a common observation that the sensitivity and specificity estimates differ among validation studies, which can be explained due to differences among reference population and sampling strategies [8]. Differences in sensitivity and specificity between diagnostic methods can result in a considerable variation in prevalence estimations, when they are not taken into account. For this reason, reliable estimates of sensitivity and specificity of diagnostic tests are necessary.

Various methods have been described for detection of antibodies against enteropathogenic *Yersinia* in serum samples of pigs at farms and in juice extracted from tonsils and meat at farms and slaughterhouses [9–14]. However, these diagnostic tests have different sensitivities and specificities making the direct comparison of the results difficult.

The true prevalence can be estimated from an apparent prevalence by using frequentist or Bayesian methods. For example, frequentist methods assume that true prevalence is a fixed unknown quantity by which a randomly chosen individual from the population is infected [7]. One of the estimators of true prevalence is the Rogan-Gladen estimator [15]. The Bayesian inferences have been advocated as more flexible and useful to solve complex problems [16], and they allow the incorporation of prior information in addition to the data. The Bayesian approach has been used in validation

of diagnostic methods, providing a reliable estimate of the sensitivity and specificity when there is more than one diagnostic test but no gold standard. An example of this is the evaluation of the diagnostic test for detection of classical swine fever [17]. Also, a Bayesian hidden variable model has been developed to study the occurrence of foodborne pathogens in the pork production chain [18].

The true prevalence of *Y. enterocolitica* in pigs sampled in farms and slaughterhouses is not directly noticeable. These should be estimated using the information from the apparent prevalence and the sensitivity and the specificity of the diagnostic test [7]. Neither the sensitivity nor the specificity of the commonly used tests is known with certainty, which introduces additional uncertainty when adjusting apparent prevalence. Using a Bayesian analysis, the true prevalence of enteropathogenic *Yersinia* in serum of Finnish pigs has been estimated. The sensitivity and specificity of the diagnostic test were also estimated.

2. Materials and Methods

2.1. Definitions. Definitions of prevalence, sensitivity, and specificity were considered as defined by Greiner and Gardner [8] and Thrusfield [19]. Apparent prevalence (Ap) is the proportion of the pig population that tests positive using a diagnostic method, and true prevalence (Tp) is the proportion of truly infected pigs in that population. The sensitivity (Se) of a diagnostic test is the proportion of infected animals that the test detects as positive. Specificity (Sp) of a diagnostic test is the proportion of noninfected animals that the test detects as negative.

2.2. Modelling Approach. The model was built in two steps using the Bayesian analysis to calculate the posterior probabilities, depending on data and prior distribution. The model estimated the true prevalence of *Yersinia* in serum samples. The prior distribution of the true prevalence was estimated based on a systematic review in the first step of the model, and later on introduced in the second step.

2.2.1. First Step. The first step is a model to estimate the prior distribution of the true prevalence and to estimate the prior distribution for sensitivity and specificity of ELISA test.

Systematic Review. The objective of the systematic review was to assess the apparent prevalence of *Yersinia* in serum samples in slaughter-age pigs and sows from farms in Finland. For this review, the questions, type of intervention, population, and outcome were used to create the inclusion criteria [20]: any study or survey that evaluates the presence of and risk factors for antibodies against enteropathogenic *Yersinia* in serum samples from slaughter-age pigs and sows in farms using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Pigtype Yopscreen, Labor Diagnostik, Leipzig, Germany).

Papers written in any language were searched, and when data was published in different articles by the same authors or in reviews, we considered them only once to avoid duplication. Data from unpublished studies was not available. The keywords used for the search were *Yersinia*, pigs or pig farms, and prevalence or seroprevalence as words in the titles or the abstracts when searching in the National Center for Biotechnology Information (NCBI) PubMed database or as the topic when searching in Web of Science. We also looked over the reference lists of the relevant papers and in auxiliary data sources, such as the Google search engine.

All studies identified were assessed against the defined inclusion criteria. Selection of studies was carried out in two stages: the first stage by screening the title and abstract of the manuscripts and the second stage by screening the full text. The number of publications selected from the systematic review was 4, while 8 manuscripts were excluded from the review because they failed in at least one of the inclusion criteria (the list of manuscripts is shown in Table 1); for example, the diagnostic tests described in the manuscripts were different from the commercial ELISA kit, and thus the sensitivities and specificities, or the samples were taken at the slaughterhouses.

The data collected from each of the manuscripts was as follows: the number of positive pigs and the number of positive farms (or herds), the number of sampled pigs and the number of sampled farms (or herds), age of sampled pigs, methodology used for analysing the samples, when and where (country level) the study was carried out, the authorship, and the published journal. We took into account data taken from tables when there was any inconsistency between data of the text and the tables. Data was collected from the selected studies and recorded in Excel (Microsoft Corp., Redmond, WA).

Construction of the Model with Literature Data. Information on number of positive pigs (or herds) and number of sampled pigs (or herds) obtained from the systematic review was used as observed data. Noninformative (uniform) prior distributions Beta(1, 1) were assigned as the prior distributions of pig and herd level true prevalence in the literature data, since it is commonly used as prior distribution for binomial proportions [29] when the prevalence is a random variable. As result, the posterior distributions of the prevalence, based on literary data, were used as informative prior distributions in the second stage below. In this way, the information from previous literature becomes utilized, with the assumption that the selected collection of literature represents roughly similar prevalence in pig populations in Finnish studies.

Information provided by the validation report published by the manufacturer of ELISA test (Pigtype Yopscreen, Labor Diagnostik, Leipzig, Germany) was used to estimate the prior distributions for sensitivity and specificity of the serological analyses. The sensitivity of ELISA was modelled using the validation report of the manufacturer, where x out of n infected animals tested positive; then beta$(x + 1, n - x + 1)$ gives the posterior distribution of sensitivity, assuming a binomial model and uniform prior distribution for sensitivity [30].

Estimates of the pig and herd prevalence were calculated in this first step by using a model mathematically similar to the one use in the second step. The obtained posterior

Table 1: List of papers selected from the systematic review to obtain the prior estimates of pig and herd level prevalence of enteropathogenic *Yersinia* in Finland and the list of papers from the systematic review that were excluded.

Author	Reference	Sample (location)	Country	Number of positive pigs/number of sampled pigs (%)
Included				
Vilar et al. [21]	Foodborne Pathog. Dis., 2013, 10: 595–602	Serum and faeces (farm)	Finland	182/334
Virtanen et al. [14]	Appl. Environ. Microbiol., 2012, 78: 3000–3003	Serum and faeces (farm)	Finland	31/65
Von Altrock et al. [11]	Berl. Munch. Tierarztl. Wochenschr., 2006, 119: 391–396	Serum and faeces (farm)	Germany	573/900
Von Altrock et al. [13]	Foodborne Pathog. Dis., 2011, 8: 1249–1255	Serum (farm and slaughter)	Germany	574/900
Not included				
Vanantwerpen et al. [9]	Prev. Vet. Med., 2014, 116: 193–196	Meat juice (slaughter)	Germany	4652/7047
Meemken et al. [22]	Prev. Vet. Med., 2014, 113: 589–598	Meat juice (slaughter)	Germany	1805/3323
Stojek et al. [23]	Bull. Vet. Instit. Pulawy., 2010, 54: 309–313	Serum (farm)	Poland	39/226
Nesbakken et al. [24]	Emerg. Infect. Dis., 2007, 13: 1860–1864	Serum and faeces (farm)	Norway	27/1073
Nesbakken et al. [25]	Int. J. Food Microbiol., 2006, 111: 99–104	Serum and faeces (farm)	Norway	163/239
Nesbakken et al. [26]	Int. J. Food Microbiol., 2003, 80: 231–240	Serum (slaughter)	Norway	21/24
Thibodeau et al. [27]	Vet. Microbiol., 2001, 82: 249–259	Serum and faeces (slaughter)	Canada	192/291
Skjerve et al. [28]	Int. J. Food Microbiol., 1998, 45: 195–203	Serum (slaughter)	Norway	1774/4029

medians and 95% PI (probability interval) of the prevalence were used as inputs in the software Betabuster (downloaded from http://www.epi.ucdavis.edu/diagnostictests/betabuster.html) to obtain the shape parameters for the prior beta distributions to be introduced in the second step of the modelling. When the estimated value was between 0 and 0.5, the 95th percentile was chosen, and when the estimated value was between 0.5 and 1 the 5th percentile was chosen, according to the instructions provided by the copyright holders of Betabuster. The beta prior distribution of the specificity was also obtained using this procedure.

2.2.2. Second Step. The second step is a model to estimate the pig and herd true prevalence of *Yersinia* in serum samples in Finland.

Collection and Analyses of Samples. The study was carried out in Varsinais-Suomi region that accounts for 28% of the total pigs in Finland (pig census from Matilda, Agricultural Statistics of Ministry of Agriculture and Forestry, 2010). The number of pigs to be sampled was calculated as previously described by Vilar et al. (2013) [21]. Individual serum samples from 120 slaughter-age pigs (50 kg or more) and 107 sows were collected in 16 farms and analysed for occurrence of antibodies against *Yersinia*. The total number of sampled pigs and the number of pigs positive using the diagnostic test in each farm were recorded to calculate the prevalence at pig and herd level.

Serum samples were tested for the presence of *Yersinia* antibodies by using a commercially available ELISA kit (Pigtype Yopscreen, Labor Diagnostik, Leipzig, Germany), with a cut-off optical density (OD) value of 0.2 according to the manufacturer's instructions.

Construction of the Model with Observed Data. A binomial sampling model was assumed as the population size in each farm was large enough compared with the sample size. The size of the farms was on average 630 slaughter-age pigs and 306 sows. The average number of slaughter-age pigs sampled in each farm was 13, and the average number of sows sampled in each farm was 9. The Bayesian model to estimate true prevalence was mathematically constructed from the conditional distributions (shown in Figure 1):

$$
\begin{aligned}
&x[i] \mid \mathrm{Ap}[i], n[i]; \sim \mathrm{Bin}(\mathrm{Ap}[i], n[i]),\\
&\mathrm{Ap}[i] \leftarrow \mathrm{Tp}[i] * z[i] * \mathrm{Se} + (1 - \mathrm{Tp}[i] * z[i]) * (1 - \mathrm{Sp}),\\
&\mathrm{Tp}[i] \sim \mathrm{beta}(\alpha\mathrm{Tp}, \beta\mathrm{Tp}),\\
&\mathrm{Se} \sim \mathrm{beta}(\alpha\mathrm{Se}, \beta\mathrm{Se}),\ \mathrm{Sp} \sim \mathrm{beta}(\alpha\mathrm{Sp}, \beta\mathrm{Sp}),\\
&z[i] \sim \mathrm{dbern}(\mathrm{tau}),\ \mathrm{tau} \sim \mathrm{beta}(\alpha\mathrm{tau}, \beta\mathrm{tau}),\\
&\mathrm{Tp0}[i] \leftarrow \mathrm{Tp}[i] * z[i],
\end{aligned}
$$

where $x[i]$ is the observed number of pigs that tested positive in farm i, and $\mathrm{Ap}[i]$ is the probability that a randomly selected pig from farm i tests positive, and $n[i]$ is the sample at that farm. $\mathrm{Ap}[i]$ is the apparent prevalence in each farm. $\mathrm{Tp}[i]$ is the true prevalence for a truly positive farm, that is, prevalence when there is at least one truly positive animal. Since a farm can be truly nonpositive, the actual true prevalence is $\mathrm{Tp0}[i] = \mathrm{Tp}[i] * z[i]$, where $z[i]$ represents an indicator variable that a farm is truly positive; that is, at least one animal would be truly positive. The actual true prevalence $\mathrm{Tp0}[i]$ for a farm is effectively described as a zero inflated distribution with a point probability mass at zero. The z variable is also needed for the correct interpretation of Tp as the true prevalence for pigs in infected farms, because also the prior of Tp is based on previous data providing posterior distribution of true prevalence in infected herds.

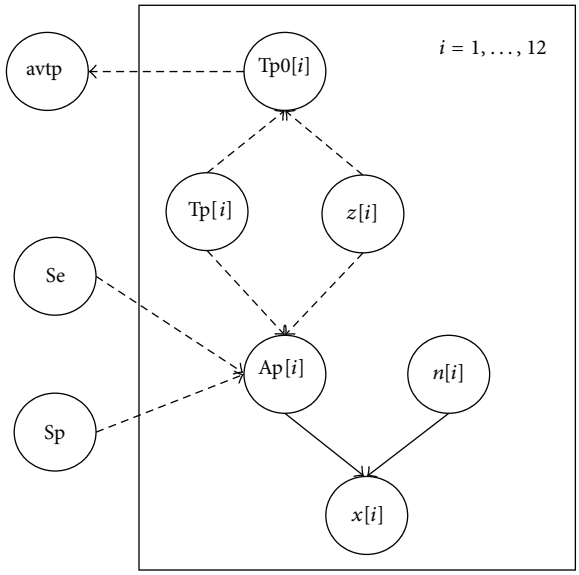

Figure 1: Graphical model used for the Bayesian analyses to estimate the true prevalence, presenting the conditional dependency structure between variables. The observed variable, $x[i]$, is the number of pigs detected positive (Ap) in the sample of size $n[i]$. The priors are beta distributions for the sensitivity (Se) and specificity (Sp) of the diagnostic test. Tp$[i]$ is the true prevalence, for a truly positive farm. Since a farm can be truly nonpositive, the actual true prevalence is Tp0$[i]$ = Tp$[i] * z[i]$, where $z[i]$ represents an indicator variable that a farm is truly positive.

There is variation between farms in true prevalence so that it is not realistic to assume a common prevalence for all farms. These differences are accounted by modelling prevalence as a farm specific parameter. Finally, tau represents herd true prevalence, the proportion of truly positive herds. The prior of tau was also calculated based on the literary review. The independent beta prior distributions obtained in the first step of this paper were used to take into account the uncertainty in the prevalence as well as in the diagnostic test sensitivity and specificity [31]. Thus, the priors for prevalence were based on the literature data, expressed as beta distributions, beta(αTp, βTp), conditionally based on that the population is infected.

Bayesian analysis was also used for upscaling estimates for a larger finite population, assuming that it is similar to the study population. Data of pig census was obtained from Matilda (Agricultural Statistics of Ministry of Agriculture and Forestry, 2010) and used to calculate the apparent and true prevalence of *Yersinia* in the whole of Finland. The upscaling was based on evaluating the average of actual true prevalence in the study farms avtp = mean(Tp0$[1, \ldots, 12]$), which represents the actual true prevalence in the study population of pig herds. Assuming that these are representative of all herds in the census, the expected number of positive pigs is avtp times the census size. Posterior distribution of this was computed.

Models were constructed in WinBUGS 1.4.3, and the graphical representation is shown in Figure 1. Inferences were based on 50000 iterations after a burn-in for convergence of 1000 iterations. Results of the posterior probability distributions are summarized by the median and the probability intervals (PI).

2.3. Sensitivity Analysis. Different prior distributions and noninformative prior distributions were used to perform the sensitivity analysis. Different prior distributions for the prevalence were used in the set of priors 1. Noninformative prior distributions for prevalence and sensitivity were introduced in the set of priors 2. Later on, the posterior median values obtained were compared for significant differences by a general linear model for repeated measures.

3. Results and Discussion

In this study a Bayesian analysis was used to provide reliable information on the prevalence of enteropathogenic *Yersinia* in pigs sampled at farms in Finland, and also to provide useful and relevant information of the diagnostic test commonly used for their detection. Table 2 shows the estimates of the posterior distributions of the pig and herd true prevalence from the model built in the first step. The sensitivity and the specificity of the diagnostic tests are also shown. These values were used when building the model of the second step. The prior distribution of the sensitivity of the commercial ELISA used to test the serum samples for the presence of *Yersinia* antibodies was beta(63, 29), and the specificity prior distribution was Sp ~ beta(6.0, 1.1).

The results of the posterior probabilities obtained in the second step are shown in Table 3. The posterior probability of the true prevalence of enteropathogenic *Yersinia* in slaughter-age pigs had a median value of 67.5%. The predicted total number of *Yersinia* positive slaughter-age pigs was 329,000 (308,400–345,800) out of 487776 slaughter-age pigs in the whole of Finland. The posterior probability of the true prevalence of enteropathogenic *Yersinia* in sows had a median value of 74.0%. The true prevalence of enteropathogenic *Yersinia* in serum samples from slaughter-age pigs estimated in the present study was lower than apparent prevalence reported previously [14, 21, 32–38]. However, those studies were based on a frequentist approach. On the other hand, when there is no prior information, frequentist analysis produces good estimates of prevalence [39], and this would correspond to Bayesian analysis with noninformative priors. However, some background information about sensitivity and specificity is needed in both cases.

Table 3 also presents the sensitivity analysis conducted by comparing the model with the original set of priors with the other sets of priors. Sensitivity analysis serves to illustrate how prior knowledge could affect the posterior estimates [40]. Although the values were not exactly similar, no significant differences were found between posterior medians and their PI. The model used was not very sensitive to the choice of priors, as the posterior probabilities for the three sets of priors were similar across the pig populations.

It has been reported that prevalence is associated with the prevalence of *Yersinia* in tonsils [10] and in faeces [11, 21]. However, the prevalence values of *Yersinia* are usually higher than the prevalence values of *Y. enterocolitica* in faeces,

Table 2: Estimates of the posterior distributions of the prevalence of enteropathogenic *Yersinia* in serum at pig and herd level. Estimates were obtained based on a systematic review of the literature and used for building the model in the second step.

Sample	Parameter	Median (95% PI)	Beta distribution	
			Alpha	Beta
Serum	Herd prevalence	0.879 (0.418–0.994)	4.157	1.435
	Pig prevalence slaughter-age pigs	0.883 (0.694–0.992)	16.461	3.049
	Pig prevalence sows	0.901 (0.469–0.995)	4.779	1.415
	Sensitivity[a]		63	29
	Specificity[b]	1	6.024	1.051

PI: the 95% probability intervals.
[a]Information obtained from the validation report of the commercial ELISA test.
[b]Specificity value of 1 was replaced by a most likely value of 0.9.

Table 3: Probability posterior estimates for the prevalence of enteropathogenic *Yersinia* in serum at pig and herd level. Sensitivity and specificity for the diagnostic test are also shown. Estimates with different set of priors are also presented, as part of sensitivity analyses.

Sample	Parameter	Posterior estimates, median (95% probability interval)		
		Original model	Set of priors 1	Set of priors 2
Serum	Sensitivity[a]	0.795 (0.736–0.848)	0.802 (0.744–0.855)	0.919 (0.833–0.990)
	Specificity[a]	0.969 (0.853–0.999)	0.961 (0.826–0.998)	0.978 (0.894–0.999)
	Herd prevalence slaughter-age pigs	0.776 (0.522–0.937)	0.782 (0.528–0.943)	0.777 (0.522–0.936)
	Herd prevalence sows	0.868 (0.625–0.981)	0.868 (0.628–0.979)	0.869 (0.634–0.978)
	Pig prevalence slaughter-age pigs	0.675 (0.632–0.709)	0.645 (0.572–0.709)	0.654 (0.613–0.689)
	Pig prevalence sows	0.740 (0.573–0.824)	0.749 (0.586–0.829)	0.720 (0.578–0.803)

[a]Estimates calculated considering all pigs, that is, slaughtered-age pigs and sows.
Herd prevalence and pig prevalence are based on the estimates of tau and Tp, respectively.
The following are other priors for sensitivity analysis.
Set of priors 1: using different prior distributions for pig prevalence.
Set of priors 2: using noninformative prior distributions beta(1, 1) for pig prevalence and for sensitivity beta.

both collected in farms [21]. This difference can be explained because the antibodies are usually present long after an infection starts [10, 41] and because the commercial ELISA test used in the present study detects antibodies based on the outer membrane proteins and thus detects infections with all pathogenic *Yersinia*. However, in Finland the prevalence of *Yersinia pseudotuberculosis* has been reported to be less than 8% [42, 43].

Sensitivity and specificity of the ELISA diagnostic test were 79.5% and 96.9%, respectively. The estimations obtained indicated that the commercial ELISA test, although good, had lower sensitivity and specificity than that previously reported by the manufacturer. Some studies [11–13] have used the commercial ELISA test but the accuracy characteristics of 100% sensitivity and 100% specificity reported by the manufacturer have not been discussed. Furthermore, no tests can be considered as having both 100% sensitivity and 100% specificity, as it is thought that estimates vary among validation studies, such as sampling strategies, technical variation between laboratories, choice of gold standard, and state of infection [8].

4. Conclusions

By using the estimates obtained by the Bayesian analysis it was possible to estimate the true prevalence of *Yersinia* in the population under study, without sampling all animals. Consequently, the model constructed in the present study can be extended when studying a country's population, which would overcome the logistic difficulties of sampling high numbers of animals. The Bayesian approach provided a reliable estimate of the sensitivity and the specificity of the commonly used commercial ELISA for detection of enteropathogenic *Yersinia*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Possible Use of Bacteriophages Active against *Bacillus anthracis* and Other *B. cereus* Group Members in the Face of a Bioterrorism Threat

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Anthrax is an infectious fatal disease with epidemic potential. Nowadays, bioterrorism using *Bacillus anthracis* is a real possibility, and thus society needs an effective weapon to neutralize this threat. The pathogen may be easily transmitted to human populations. It is easy to store, transport, and disseminate and may survive for many decades. Recent data strongly support the effectiveness of bacteriophage in treating bacterial diseases. Moreover, it is clear that bacteriophages should be considered a potential incapacitative agent against bioterrorism using bacteria belonging to *B. cereus* group, especially *B. anthracis*. Therefore, we have reviewed the possibility of using bacteriophages active against *Bacillus anthracis* and other species of the *B. cereus* group in the face of a bioterrorism threat.

1. Introduction

Shortly after the September 11 terrorist attacks using captured planes, letters containing anthrax spores were posted to news media and the US Senate. Five people died and several others survived the disease. This incident proved that bioterrorism using *B. anthracis* is a real danger and society needs an efficient weapon to neutralize this threat. Experts believe that if such an attack occurred in a large city, hundreds of thousands of people could be at risk of the deadly disease, while the present systems of defense are insufficient.

Anthrax is an infectious disease with high epidemic potential (characterized by high morbidity and mortality, with real possibility of being used in a bioterrorism attack with spores of *B. anthracis*). The pathogen (especially spores) that is the cause of anthrax may be transmitted in human populations by way of aerosolization (natural or artificial), resulting in epidemics with high mortality [1]. These bacteria are rare in the USA and highly infective and pose a huge threat to public health [2]. There is no strictly defined infectious dose for humans. Its amount may be influenced by such factors as the route of infection (type of anthrax), state of health of the infected person, and virulence of the infecting strain [3]. The disease caused by *B. anthracis* is treatable with antibiotics (such as penicillin G, amoxicillin, or ciprofloxacin), but the prognosis depends on the time after which the pathogen is identified and the application of appropriate therapy [4].

Anthrax spores are easy to store, transport, and disseminate and may survive in soil for many decades. Due to this feature, *Bacillus anthracis* is likely to be used as a bioterrorist weapon [5]. Moreover, the bacteria produce dangerous

toxins. Antibiotic resistance in bacteria from environmental samples may cause serious problems in treating anthrax. The capsule that can be present in some strains could be dangerous as it inhibits phagocytosis of these bacteria. Moreover, the *B. cereus* group—which *B. anthracis* belongs to—consists of very homogeneous bacteria with close relatedness. This feature may pose problems with identification of and differentiation between bacteria belonging to this taxonomy group.

Bacterial viruses, bacteriophages (phages), are natural enemies of bacteria and recent data strongly suggest their effectiveness in treating bacterial diseases including those caused by antibiotic-resistant microbes [6]. Their biology and current applications have been recently summarized in detail [7]. Therefore, it is clear that phages should also be considered a potential tool against bioterrorism using *B. anthracis* or other *B. cereus* group bacteria.

2. *B. anthracis* as a Bioterrorism Tool

2.1. Pathogenesis of B. anthracis and Other B. cereus Group Bacteria. The etiological agent of anthrax, *Bacillus anthracis*, is a Gram-positive, aerobic or facultatively anaerobic, spore-forming, and rod-shaped bacterium, which appears in cell chains. *B. anthracis*, *B. anthracis*, together with four other species (*B. cereus* sensu stricto, *B. thuringiensis*, *B. mycoides*, and *B. weihenstephanensis*) constitute the *Bacillus cereus* group [8]. This zoonotic pathogen is mainly present in soil, water, and animals. It infects animals and, occasionally, humans [5, 9]. There is a risk of passive transfer of anthrax from animals to humans through insects [5]. Spores produced in the presence of oxygen [10] are stable and resistant to harsh external conditions like heat, cold, pH, desiccation, and chemicals. They can germinate when exposed to a nutrient-rich environment, such as the tissues or blood of an animal or human host [11]. The climate may directly or indirectly influence the way in which an animal comes into contact with the spores or affect the general state of a host's health and the level of their resistance to infection [5]. There are data [5] regarding the impact of various factors, such as rainfall, temperature, state of the host, and population density, on the epidemiology of anthrax; however, there is no agreement on the roles played by these factors in the incidence of the disease. Unfortunately, there are no hard scientific data to support these theories.

In humans there are three main forms of this disease, cutaneous, gastrointestinal, and inhalational, according to the route of infection [12]. Each of these forms can be lethal. The most dangerous is the inhalation form (it may be induced by 8×10^3–5×10^4 spores). In people untreated, death occurs in 97 to even 100% of cases within 3–5 days, but in people treated already at the early stage of the disease the mortality rate is reduced to 75%. On the other hand, mortality in untreated cutaneous form of anthrax can range from 10 to 20%, and in treated cases it is below 1% [4, 13].

The virulence of anthrax is associated with the production of poly-D-glutamic acid polysaccharide capsule (PDGA) [14, 15]. The *cap* gene encoded on the pOX_2 plasmid is responsible for the synthesis of the capsule. The mechanism of inhibition is not well established [16]. One of the possibilities may be phagosomal escape. Moreover, it is suggested that the capsule may block bactericidal activities of neutrophil cationic peptides, for example, α- and β-defensins. It has been demonstrated that the capsule is poorly immunogenic and evades recognition as an antigen by the immune system because it protects the surface antigens and protects bacterial cells from the circulating antibodies, therefore enabling the spread of bacilli inside the host body [17]. Moreover, *B. anthracis* strains produce toxins that consist of three peptides: protective antigen (PA), lethal factor (LF), and edema factor (EF). These peptides are conditioned on the pOX_1 plasmid where three genes, *pag*, *lef*, and *cya*, are located. The LF protein in combination with PA creates a lethal toxin, but EF with PA forms an edema toxin. Anthrax toxins are produced by vegetative forms of bacteria. In combination, virulence factors promote the multiplication of bacilli after invading the human organism. The lethal toxin causes the release of tumor necrosis factor (TNF) and interleukin-1 (which are responsible for rapid health deterioration during the inflammatory process) from macrophages, as well as development of symptoms and, possibly, cell damage [18, 19]. The edema toxin causes the formation of edema in tissue as a result of water and Cl^- ions loss from cells and may inhibit neutrophil phagocytic activity and oxidative burst [18]. Anthrax can also result in necrosis, septicemia, organ failure, and death. If not treated, patients may die in a few days.

Initially, the symptoms of anthrax are nonspecific (symptoms are difficult to distinguish from those of other diseases); therefore, it is difficult to recognize and quickly apply proper treatment [3]. However, at the initial stage of the disease, people should be treated with antibiotics or vaccinated as early as possible, as progression of the disease (especially in the case of inhalational anthrax) is rapid, and if the treatment is not applied within the first 24 h from first observed symptoms, it may result in death.

In some cases, the consumption of contaminated food (meat and milk) has led to foodborne illnesses associated with *B. anthracis* [20]. Conversely, the closely related species *B. cereus* is responsible for the majority of foodborne illnesses attributed to the *B. cereus* group. There is a broad range of foods associated with *B. cereus* infection including food of both animal and plant origin. Many of these foods may contain *B. cereus* since spores of this organism are heat-resistant and can survive cooking [20]. Food poisoning by *B. cereus* is a result of food-contaminating enterotoxins—emetic (vomiting) and diarrhogenic—that are produced by the bacteria. The first toxin causes intoxication as a result of thermostable toxin (cereulide, cyclic peptide toxin) ingestion, while the second, diarrheal one is an effect of infection by vegetative cells or spores producing heat-labile enterotoxin in the small intestine [21]. The symptoms of emetic poisoning occur within 1–5 h after ingesting contaminated food. This toxin is produced during bacterial growth in food [22]. Strains that are able to cause diarrhea are difficult to identify because of the diverse and complicated mechanisms characterizing this

type of infection. The symptoms of diarrheal syndrome occur 8–16 h after food ingestion [23].

2.2. Epidemiology of B. anthracis and Other B. cereus Group Bacteria. Nowadays, the risk of anthrax is extremely small, at least in developed countries, where animal husbandry is carried out in modern conditions and hygiene is respected. Anthrax may constitute a problem especially in countries where the vaccination of animals is not practiced. However, the risk of bioterrorism using the pathogen is also a real threat.

There is no strictly defined infectious dose for humans. Its amount may be influenced by such factors as the route of infection (type of anthrax), state of health of person, and virulence of the infecting strain [3]. The infectious median lethal dose (LD50) is likely within the range of 2500–55000 spores [24]. But there are data indicating that for induction, cutaneous anthrax 10 or fewer spores are required [25]. In the case of gastrointestinal anthrax, however, the defined minimal infectious dose (Mid50) is estimated to be approximately 10^{11} spores [26]. Epidemiological evidence suggests that the majority of cases of foodborne illness caused by *B. cereus* have been associated with concentrations in excess of 10^5 cfu/g in food. Only rare cases of illness involving 10^3–10^5 cfu/g of *B. cereus* in food have been reported [27]. Both *B. cereus* and *B. anthracis* bacteria may infect people, but more serious side effects may be observed in immunocompromised, young or old patients in particular [20]. For example, skin injuries may be a convenient way of anthrax spreading; for example, soil contaminations may be dangerous especially for patients predisposed to bacterial infections, such as those suffering from diabetic foot syndrome, because of nonhealing ulcers that may constitute the way for spores or vegetative forms to invade the human organism.

Natural *B. anthracis* is present in the environment; for example, the highest level of anthrax spores has been detected in Namibia, where in the vicinity of animal carcasses it amounted to 1 000 000 spores per 1 g of soil [28]. Using anthrax bacilli for bioterrorism purposes requires much higher doses [2]. Data show that 100 kilos of powdered spores may be a lethal dose for 10^{13} people.

A simulation of an expert committee of the World Health Organization [5] showed that the release of 50 kilos of anthrax spores over a city would result in 250 000 infections leading to 95 000 deaths (without treatment). The cost of a bioweapon attack using anthrax was estimated at $26.2 billion per 100 000 people exposed to the biowarfare agent [24]. Turnbull et al. indicated that the highest levels of anthrax spores (20 to 40 colony-forming units of spores per cubic meter were detected) were found in air at dusty anthrax carcass sites in Namibia, 3 to 9 m above those sites [5]. Interestingly, the results of estimation indicated that it would take about 2.5 minutes for a human to inhale 1 spore of *B. anthracis*. But the authors suggested that the probability of inhaling anthrax spores depends significantly on the size of the particles to which spores are attached [3].

The Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological (Biological) and Toxin Weapons of 1971 prohibits conducting research using bacteria (e.g., *B. anthracis*), their toxins, and viruses, for offensive purposes. It does, however, permit the development of vaccines for defensive purposes. There are speculations yet that somegovernments fund the conduct of research concerning *B. anthracis* application as a biowarfare agent [29]. The epidemic in the Sverdlovsk military laboratory (1979) was caused by accidentally releasing aerosol containing anthrax spores (probably 1-2 g), which were carried by the wind and which caused the greatest ever documented epidemic of pulmonary anthrax in human history. Moreover, *B. anthracis* could also be involved in cases of unintentional spread of bacteria, as recently happened when a laboratory mix-up exposed many employees to anthrax [30]. The 2012 report showed that decontamination after the anthrax letters attacks from 2001 in the US, as a result of which 11 cases of anthrax inhalation (five patients died) and 11 cases of cutaneous anthrax were reported, costed $320 million [31].

B. anthracis may be attractive as a biological weapon due to low production costs and ease of transmission [2]. Vegetative *B. anthracis* forms are not easily transmitted, but spores can be transmitted to humans, and therefore applying spores in the aerosol form is probably the most effective. The anthrax bacillus is easy to obtain in culture and the costs of spore production are low; it is estimated that the production of 1 kilo of spores averages $50 [2, 32]. The source of infection may be anthrax spores contained in aerosol or foods. After release, the anthrax aerosol is odorless and invisible and may be transferred over a long distance (many kilometers). Spores are robust and long-lasting (they are resistant to heat, chemicals, ionizing radiation, and ultraviolet light) [19]; for example, spores that were isolated in Kruger National Park in Africa from animal bones were estimated to survive about 200 years [33]. Boiling spores in water for 10 minutes causes their complete destruction [18]. Bacteria that belong to the *B. cereus* group are widespread and able to form spores which have the ability to remain resistant despite long-term storage and show thermostability. These are the reasons for the existence of a wide variety of foodborne illnesses.

B. anthracis is usually a drug-sensitive strain, but strains that may be multidrug resistant are deliberately engineered [4]. A potential *B. anthracis* terrorist attack may be caused by contamination of food and water, spread by letters, or spraying in public transport. It may cause widespread panic and requires special, quickly arranged actions for collective health preparedness. Results of anthrax attack simulation demonstrate that aerosol spores penetratethroughout a building in less than 4.5 min [34]. What is more is that prompt action, such as closing the doors and windows, shutting the ventilation system, and deactivating heating or air conditioning, would effectively reduce spore concentration inside the site in which the aerosol was released [35].

3. *B. anthracis* Bacteriophages

Bacteriophages (phages) are viruses that infect and multiply only in bacterial cells. It is estimated that their abundance in the biosphere exceeds $10^{30\text{-}31}$ virions [36, 37], ten times

more than bacterial cells [38]. Bacteriophages are present in the environment: soil, marine water [39], and extreme conditions such as the Sahara desert sands or hot springs [37, 38]. We consume them with food and drinking water. Together with bacteria they constitute an integral part of the microbiome [40]. Phages in humans may be successfully used in the treatment of a wide range of infections, both local and systemic [6]. Applying phage therapy is safe for patients. There has been low incidence of phages' adverse effects (e.g., nausea, loss of appetite, superinfection, and body temperature increase) associated with the use of them [6, 41]. The results obtained by Łusiak-Szelachowska et al. (2014) indicated that the induction of antiphage antiserum activity in patients receiving phage therapy does not influence the final outcome of the therapy [42]. Despite these data, phage therapy (regarding the use of different phages or different cocktails consisting of different phages) has not been approved by the FDA so far. Clinical trials that may confirm the safety and effectiveness of the therapy need to be conducted [6].

Interestingly, bacteria's resistance to antibiotics does not contribute to the formation of phage resistance [43]. Therefore, phages may be used to treat infections caused by antibiotic-resistant bacterial strains, for example, methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and extended-spectrum beta-lactamases producing strains (ESBL) [44–48]. Moreover, phages may be simultaneously active against bacteria resistant to many antibiotics [47, 49].

Anthrax-specific phages were first isolated in the 1950s [50]. In 1951 McCloy isolated the lysogenic W phage from an atypical *B. cereus* strain [51]. The phage was specific to all 171 isolates from *B. anthracis* but showed limited activity against *B. cereus* strains (only 2 of 54 strains) [52]. These data indicate specific activity of the phage, especially against anthrax bacilli. Then, Brown and Cherry isolated a gamma (γ) phage which was the lytic variant of the W phage [53]. They demonstrated that the γ phage is able to lyse both encapsulated and nonencapsulated *B. anthracis* strains. However Negus et al. suggested that the optimized capsule production in *B. anthracis* tested by Brown and Cherry might have been carried out incorrectly [54]. Interestingly, the phages tested by Negus et al.—γ, Fah, F7, and F9—were able to lyse *B. anthracis* Sterne in both capsulated and nonencapsulated form.

Phages active against *B. anthracis* (both lytic and lysogenic) are widespread in the environment and have been isolated from soil, carcasses, feces, sewage, and the intestinal tract of the earthworm *Eisenia fetida* [54–58]. *B. anthracis* specific phages and their characteristics are presented in Table 1. Apart from phages listed in Table 1, there have been many more anthrax phages isolated, for example, Nk, DB, and SP50, belonging to *Myoviridae* isolated from Iowa topsoil [55]; BA39, BA21, BA28, and BA51 isolated from a sewage treatment plant (Germantown), belonging to *Myoviridae* [59]; ϕ20 lysogenic phage induced by exposure to UV light, belonging to *Siphoviridae* [60]. Interestingly, Lee et al. prepared a review in which they characterized and collected three groups of bacteriophages infecting members of *B. cereus* group, according to their genomic analysis [61].

Bacteriophages, as well as lysins (encoded in phage genomes), could be useful in the treatment of infections caused by *B. anthracis*, destruction of *B. anthracis* germinated spores, and environmental disinfection. Treatments with phages or lysins may be extremely important because of being potentially safe for humans infected with anthrax and threatened with death caused by those bacilli. Bacteriophage-based methods for identification and/or treatment of anthrax may be methods of the future. This common and well-investigated tool—which bacteriophages constitute—is also very useful in molecular biology. There are methods of *B. anthracis* strain identification, for example, the γ test approved by the FDA in 2005 [1] or the bioluminescence test based on light detection after the application of a phage with the lux AB gene. Possible phenotypic alterations of temperate phages in *B. anthracis* include an influence on bacterial sporulation (the prophage state may induce rapid sporulation phenotype), biofilm formation, and induction of exopolysaccharide production [69]. However, bacteriophages may be used not only as antiterrorism tools, but also aspotential bioterrorism agents [70]. For example, lysogenic bacteriophages that contain virulence or drug resistance genes may be used for genetic manipulation, enabling the modification of nonpathogenic bacteria into a strain that would be resistant to available antimicrobial drugs. Despite the intensive studies on isolation and characterization of *B. anthracis* phages, many questions still remain unanswered.

Apart from previously described possible phage applications, bacteriophages can be also used in controlling bacterial pathogens from the *B. cereus* group in food and food processing environments. A summary of the potential use of phages active against bacteria from the *Bacillus cereus* group, in the case of potential use of these bacteria as a biological weapon, is presented in Figure 1.

One of the advantages of bacteriophages specific for *B. anthracis* is their narrow activity against the bacterial host, being restricted to strains of *B. anthracis*—not active against closely related strains of the *Bacillus* genus (such as *B. cereus* or *B. thuringiensis*). But bacteria from the *B. cereus* group are very closely related. *Bacillus cereus* and *B. anthracis* share many common phage parasites [71]. Close relatedness between *B. anthracis* and bacteria from the *B. cereus* group enables certain phages active against *B. anthracis* to show activity also against *B. cereus* and vice versa [72].

In our opinion, when the usefulness of anthrax phages is considered a tool for therapy and the detection of bacteria, the specificity (narrow host range) is an advantage. If the aim is decontamination, the broad lytic spectrum may be helpful in case of elimination of both *B. anthracis* and other species belonging to the *B. cereus* group is the objective.

4. Identification of *B. anthracis*

Should a bioterrorism attack occur, there must be a possibility for its rapid detection and identification in an average microbiological laboratory. Such work requires the biological

Table 1: *Bacillus anthracis* phages and their characteristics.

Name of phage	Type of life cycle	Short description	Phage host specificity	Possible application
Wβ	Lysogenic	Belongs to *Siphoviridae.* Inability to infect encapsulated cells [4].	Infects all 171 tested nonencapsulated strains [50], but does not infect other *Bacillus* strains.	Preparing bioluminescent reporter bacteriophage for *B. anthracis* **detection** in clinically relevant samples [56] and providing an antibiotic susceptibility profile [4].
Gamma phage (γ)	Lytic	Belongs to *Siphoviridae* [57]. Cannot bind to GamR receptor on bacterial surface and does not encode a PDGA depolymerase. Encodes a fosfomycin resistance gene [58].	*B. anthracis* 1584; 211; SL 1809; Sterne 34F2 [51]. Not active against *B. anthracis* Ames strain that produces capsule. Strains that do not encode the pX01 plasmid are more susceptible to phage γ than strains that possess the plasmid [62].	**Identification** of *B. anthracis* strains and its differentiation from other similar strains from *B. cereus* group.
AP50	Lytic	Belongs to *Tectiviridae* [55], isolated from soil. Infects only *B. anthracis* strains. Does not lyse strains belonging to different *Bacillus* spp. The lysogenic mutant AP50c is characterized by very high killing efficiency [63].	Narrow host range [64]. Lyses 33% of *B. anthracis* strains [1]. This phage may infect bacterial strains that are resistant to γ phage [63]. It does not infect the *B. cereus* ATCC4342 strain, which infects the γ phage.	Probable use **in therapy** of anthrax. It is suggested to be used in **typing and biocontrol** of *B. anthracis* [65].
Fah	Lytic	Belongs to *Siphoviridae* [66].	*B. anthracis* 1584; 211; SL 1809; Sterne 34F2 [54]. Narrower lytic spectrum. Lyses 73–89% of *B. anthracis* strains [1, 66].	Probable use **in therapy** of anthrax.
Worm intestinal phage 1 (Wip1)	Lytic	Belongs to *Tectiviridae* [67]. It was isolated from the intestinal tract of *Eisenia fetida* worms. [52].	Exhibits a narrow host range highly specific to *B. anthracis* [67]. Does not infect the *B. cereus* ATCC4342 strain, which infectstheγ phage [52].	Potentially useful diagnostic tool for efficient **identification** of *B. anthracis*; may be labelled and applied in organism for rapid readout [61].
Giraffe phage	?	Belongs to *Siphoviridae* isolated from giraffe faeces in a zoo (Long Island) [68]. This phage shows a rapid lysis phenotype.	Lyses the ciprofloxacin-resistant *B. anthracis* strain HS2-7 [68].	Possible use **in therapy** when infection is caused by antibiotic-resistant *B. anthracis* strain [67].
F7	Lytic	Isolated from bovine faeces. Belongs to *Siphoviridae* [51].	*B. anthracis* 1584; 211; SL 1809; Sterne 34F2; *B. cereus* ATCC13472; *B. cereus* ATCC *10876; B. thuringiensis* ATCC 33679 [51].	Probable use **in therapy** of anthrax.
F9	Lytic	Isolated from bovine faeces. Belongs to *Siphoviridae* [51].	*B. anthracis* 1584; 211; SL 1809; Sterne 34F2; *B. cereus* ATCC13472; *B. cereus* ATCC *10876; B. thuringiensis* ATCC 33679 [51].	Probable use **in therapy** of anthrax.
vB_BanS-Tsamsa	Lysogenic	Isolated from carcasses in Etosha National Park in Namibia. Belongs to *Siphoviridae.* Has the largest sequenced genomes of *Bacillus* siphovirus.Purified endolysin encoded in genome of this phage has broader spectrum than the phage. The largest siphovirus known to infect *Bacillus* strains [54].	Infects also strains belonging to *B. cereus* and *B. thuringiensis* [54]. Did not lyse the *B. anthracis* PAK-1 strain (resistant to both γ and cherry phage). Moderate specificity to *B. anthracis*.	Use of purified phage endolysin in *B. anthracis* **biocontrol.**

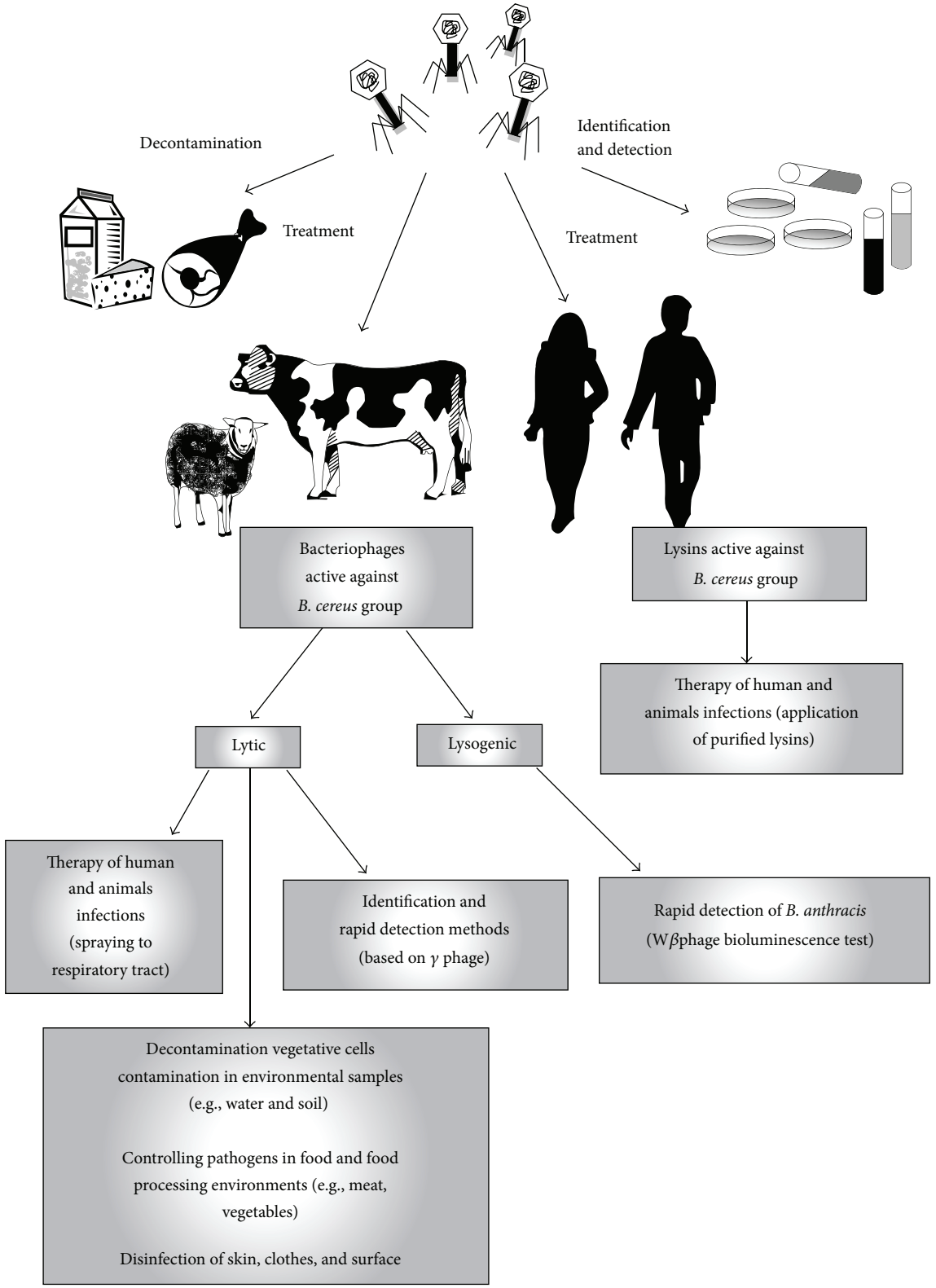

Figure 1: The possibility of using bacteriophages and lysins against bacteria from *Bacillus cereus* group.

safety level 3 use (BSL-3) [28]. To work with agents that may cause serious or potentially lethal diseases (e.g., *B. anthracis*), especially through the inhalation route of exposure, the laboratory should be designed to ensure the personnel safety. It has to, for example, be self-closing and entered through an airlock or anteroom and have double-door access and a hand washing sink near the laboratory exit; the air cannot be recirculated, and there must be negative airflow into the laboratory [73].

B. anthracis may be isolated from different animal body specimens (blood cultures, cerebrospinal fluid, stool, respiratory specimens, and cutaneous lesions), but their source depends on the type of anthrax [10].

Methods used for identifying *B. anthracis* should offer rapid detection even at low concentration of the pathogen, with no crossreactivity. They should be simple to perform and possible to perform at the site of sampling [19]. Moreover, the assay should enable the detection of both spores and vegetative forms. Therefore, the real challenge is to optimize the *B. anthracis* foolproof detection system for protecting public health.

Van Tongeren et al. studied the microbial community of the interior human environment of the International Space Station [74]. They isolated from one microbial sample multiple strains belonging to the *B. cereus* group, including *B. anthracis*. The authors emphasized that there is a real challenge in rapid detection of anthrax bacilli from that type of microbiological material.

Differentiating *B. anthracis* from other strains of the *Bacillus* genus may cause diagnostic difficulties. Standard microbiological methods used in laboratories take 24–96 h [5, 62]. Despite the introduction of the Ground Anthrax Bacillus Refined Isolation (GABRI) method to analyze environmental samples, which enables the detection of low levels of *B. anthracis* [75], this method also requires 24 or 48 h of incubation. Public security requires shorter times of detection of anthrax contamination. Methods depending on antigen detection allow the result to be obtained within several hours [19] but may show a lack of sensitivity and specificity as well as crossreaction with other strains of the *Bacillus* genus, thus giving false positive results. The nucleic acid based method (e.g., polymerase chain reaction) is highly specific but does not discriminate between live and dead bacteria [76] and clean starting samples are required [19]. So far, there is no method that can be considered reliable.

4.1. Detection of B. anthracis. Phages may have potential to be used in *B. anthracis* detection in clinical, environmental, or food samples [70]. Most phage-based assays exploit the γ phage [77]. They are based on high specificity of phages to certain bacteria species, and their detection limit is 10^3–10^5 cfu/mL. It is possible to identify anthrax bacilli as quickly as within 60–120 min.

The phage γ test is a standard method for identification of *B. anthracis* strains and differentiates them from other closely related strains from the *Bacillus* genus [78]. This routine identification test takes 2–4 days [5]. The presence of a polypeptide capsule inhibiting *B. anthracis* infection by lytic γ phage constitutes a serious problem in using this method. The synthesis of the capsule blocks the GamR receptor on the bacterial cell surface responsible for phage binding [79].

Based on the differences between phages' lytic spectrum, it is supposed that the identification/typing test using the γ phage is probably less sensitive when compared with the Wip1 phage [58, 80]. Additionally, Wip1 plaques can be detected after merely 12 h after bacterial infection.

Detection of *B. anthracis* by using Wβ phage bioluminescence enables the detection of a signal as soon as after 16 min from the moment of commencing the infection of *B. anthracis* cells with the Wβ phage possessing an incorporated luxAB reporter gene [62]. The method allows for direct detection of *B. anthracis* in clinical specimens (e.g., blood, stool, and sputum) [77] and excludes the detection of members belonging to the *B. cereus* group closely related to *B. anthracis* [4, 62]. It detects only live bacteria (~10^3 cfu/mL within 60 min) and *B. anthracis* germinated spores (within 60 min). Spores are refractory to phage because they do not show the GamR on their surface; therefore spores may be detected only in the germinating state. A higher phage titer gives a stronger detection signal. The limitations of the bioluminescent Wβ phage-based method may result from *B. anthracis*' resistance to phage infection and no possibility of the reporter phage to infect encapsulated strains. Abshire et al. found that only 2 strains out of 51 tested isolates of *B. anthracis* were resistant to the lysis caused by the γ phage [65]. It may indicate that natural phage resistance of *B. anthracis* strains is not common.

Kan et al. identified the ligand on the Wip1 bacteriophage that is highly specific to the receptor on *B. anthracis* [80]. They observed that the gene product p23 of the Wip1 bacteriophage is a receptor-binding protein on the phage surface. The presence of this protein and narrow host range of the Wip1 phage may provide new tools for the identification of *B. anthracis* strains.

The anthrax spores should be detected before the occurrence of symptoms, especially by the use of continuous monitoring of spore content in the air [81]. The system should be sensitive and selective to avoid false alarms of bioterrorist attack. Brigati et al. proposed a method based on landscape pIII phage-display libraries (that contain thousands of copies of peptides best binding to a specific antigen) and phages expressing a specific peptide used as a probe that specifically binds to *B. anthracis* spores [81]. This method is not ideal due to the possibility of clones crossreacting to other species belonging to the *Bacillus* genus. But the most specific phage display spore binding peptide EPRLSPHS bound 3.5- to 70-fold more strongly to the *B. anthracis* Sterne spores than to other strains. Also, sensors that use filamentous phages may be useful in *B. anthracis* spore identification, and wireless magnetostrictive sensors showed binding affinity to *B. anthracis* that was better than to *B. cereus* and to *B. subtilis* spores [82]. Applying filamentous phages in these methods is justified for these phages are suspected to be the most stable nucleoproteins in nature. They are extremely resistant to high temperature (even up to 80°C), acids and alkaline solutions, organic solvents (50% alcohol), and denaturing agents (6–8 mg/L urea) [81, 83]. The detection limit of the described

method is at 10^3 spores/mL [84]. Detection of anthrax spores in water using phage as a bioprobe and magnetostrictive mili/microcantilevers (MSMC) designed as a sensor platform was developed by Fu et al. [85]. This method enabled *in situ* detection. Schuch et al. prepared a rapid and highly specific system for detecting spores [86]. The detection is based on light emission in the presence of luciferin and luciferase and the release of ATP from lysed bacterial cells. It is based on the ability of PlyG to kill germinating spores and is applied using a hand-held luminometer. The signal was detected only 10 min after the addition of germinating spores of the RSVF1 strain. What is more is that the light was emitted only 5 min after adding PlyG. Moreover, a method based on the binding of *B. anthracis* vegetative cells has also been developed [87].

Shabani et al. presented a phage-modified electrode microarray method for rapid and direct impedimetric detection of *B. anthracis* [88]. It is based on the immobilization of the γ phage and its high specificity to *B. anthracis* species and provides a low-cost platform for direct identification of *B. anthracis*. Its detection limit is 10^3 cfu/mL with a sample volume of merely 40 μL.

5. Treatment of Anthrax

Without immediate treatment, inhalation of anthrax spores is usually lethal (within the first 24 h from observed symptoms, it may result in death). Therefore, therapeutic intervention should be initiated as early as possible [89]. The antimicrobial chemotherapy recommended for the treatment of patients with inhalational anthrax is effective, but long-term therapy may cause antibiotic resistance in *B. anthracis* [90]. Drugs used for postexposure prophylaxis are penicillin G, amoxicillin, doxycycline, ciprofloxacin, and ofloxacin administered for 60 days or more [24].

Penicillin has been considered the drug of choice, and it is very rare that resistance to this antibiotic is found in naturally occurring strains [9]. Ciprofloxacin, penicillin, and doxycycline are recommended for the treatment of humans and as prophylactics after exposure to the spores [91]. Many *in vitro* studies show that *B. anthracis* is susceptible to penicillins, fluoroquinolones, tetracycline, chloramphenicol, aminoglycosides, macrolides, imipenem/meropenem, rifampicin, and vancomycin [9, 91, 92]. However, the organism is resistant to cephalosporins, trimethoprim, and sulphonamides. *B. anthracis* is usually sensitive to a broad range of antibiotics. Cavallo et al. tested its sensitivity to antibiotics in 96 strains of *B. anthracis* isolated from humans (1), animals (28), and the environment (67) in France [89]. 11.5% of strains were resistant to penicillin G and amoxicillin. All of them were resistant to cotrimoxazole but susceptible to antibiotics such as doxycycline, vancomycin, clindamycin, rifampicin, imipenem, or teicoplanin.As a result of long-term antibiotic treatment *B. anthracis* strains may be converted into antibiotic resistant strains [90]. It was observed that only 11% of natural/environmentally isolated strains of *B. anthracis* were resistant to penicillin G [89].

In the case of *B. cereus*, the bacteria—due to β-lactamase production—are insensitive to penicillin-related antibiotics (merely 1% of strains are susceptible to penicillin) and show resistance to erythromycin and tetracycline, for example, carbapenem [61, 93, 94]. We suppose that, due to problems with antibiotic treatments and improvement in bacterial drug resistance, these strains may be used as potential biowarfare agents. Therefore, for public safety, there must be known an agent to which these bacteria are susceptible.

Treatment with antibiotics beginning 1 day after the exposure to an aerosol with anthrax spores can protect against death. However, optimal protection isprovided bycombining antibiotics with vaccination. Vaccination is the best form of mass protection. The first anthrax animal vaccine was developed by Pasteur in 1881. Pasteur attenuated *B. anthracis* strains and proved that these strains could protect sheep from fully virulent strains [95]. Human vaccines emerged in the middle of the 20th century [9]. Human anthrax vaccine (anthrax vaccine adsorbed, AVA), currently licensed for use in the United States and the United Kingdom, consists primarily of protective antigen (PA) absorbed onto aluminum hydroxide [96, 97]. This vaccine was tested in guinea pigs, rabbits, and rhesus macaques by Fellows et al. [98].

According to FDA prescribing information concerning the observed side effects of AVA (BioThrax), local adverse reactions have been observed (especially at injection site), for example, tenderness, pain, erythema, edema, and arm motion limitation; (≥5%) as well as systemic adverse reactions: fatigue headache and muscle aches [99]. The currently available vaccines have a chemically complicated composition and it is believed that they are insufficiently purified [100].

AVA was originally prepared for individuals in high-risk occupations, like veterinarians, farmers, and laboratory personnel working with *B. anthracis* but was also used for military personnel [96]. About 150 000–200 000 American soldiers sent in 1991 to the war in the Persian Gulf were vaccinated against anthrax [101].

The use of appropriate animal models provides better understanding of the pathogenesis of human anthrax and the development of appropriate methods of prevention and treatment. Rabbits and nonhuman primates (NHPs), for example, rhesus macaques, are commonly used as animal models of inhalational anthrax. The pathological changes observed in rabbits and NHPs are similar to those observed in humans [102]. Savransky et al. showed that the pathology caused by the inhaled form of anthrax in guinea pigs is similar to that in both rabbits and NHPs, as well as in humans. Guinea pigs have alsobeen used in anthrax vaccine studies.

Another popular animal model used to test the sensitivity to virulent *B. anthracis* is the mouse. The mouse model is useful in studies on host resistance to anthrax and on pathogenesis, how the agent establishes infection in the host, and characteristics of the spore and vegetative bacilli. It is known that different mouse strains have various sensitivities to infection by both *B. anthracis* and anthrax toxin [103]. For instance, the BALB/c mouse strain is highly resistant, and strains such as A/J and DBA/2J are highly susceptible to infection [12, 99]. Interestingly, the susceptibility of mouse strains to lethal toxin (LT) does not necessarily correlate with its susceptibility to infection. For example, the susceptibility of A/J mice to anthrax toxin appeared to differ from

the susceptibility to infection [99]. The rat and hamster, meanwhile, are important animal models for understanding the *B. anthracis* exotoxins, both LT and EF [12].

5.1. The Potential Use of Phage in Anthrax Treatment. The first phage therapy studies on *B. anthracis* were conducted by Cowles and Hale on mice [104]. The *B. anthracis* Thomas strain and bacteriophages which had been isolated from a malignant pustule, which were applied as therapeutics, were used in the experiment. The animals were inoculated (intraperitoneally) with 0.1 mL of bacteria (10^6 cfu/mL) and 0.1 mL of bacteriophage (10^9-10^{10} pfu/mL). The authors found that, only in the group inoculated with *B. anthracis* and bacteriophage mixture incubated 25 min before injection, 100% of mice survived. The results of this study also showed that only the phage, in high titer, quickly and permanently lysed the strain of anthrax used in the experiments.

Phages may be applied in phage therapy in the case of *B. anthracis* (also drug-resistant) infections [70]. For better effectiveness of therapy, phages active against *B. anthracis* should encode capsule depolymerases, to degrade the PDGA capsule that may be present in the bacterial surface. In this case phages may bind to the cell surface receptor of the bacteria and destroy these dangerous bacteria [54, 105].

Besides the whole phage particles, also endolysins can be applied in the therapy of anthrax. Endolysins are enzymes encoded in the bacteriophage genome and specifically lyse the peptidoglycan of the bacterial cell wall during the phage lytic cycle [106]. The enzymes may create new opportunities for the construction and production of genetically engineered enzymes for bacteria elimination, biocontrol, and experimental therapies. The endolysin PlyG isolated from the γ phage may be applied against *B. anthracis* (e.g., used as abiowarfare agent) [86]. Susceptibility of *B. anthracis* strains to γ phage infection and purified PlyG lysin isolated from this phage indicated that both of these agents have a narrow bacteriolytic spectrum—they especially showed high activity against almost only *B. anthracis* strains [86]. The authors decided to use isolates of streptomycin-resistant *B. cereus* RSVF1 strain because of the similarity of this strain to the *B. anthracis*. Lytic activity of lysin against this strain was the same as in the case of *B. anthracis* strains. In the study of Schuch et al., (2002) BALB/c mice were intraperitoneally infected with *B. cereus* RSVF1 (1.0×10^6 cfu/mL) and, 15 min later, treated with 50 and 150 U PlyG. The application of lysin significantly rescued mice in comparison to untreated animals. Moreover, resistance to PlyG was not observed *in vitro* in either RSVF1 or EMS RSVF1 mutagenized strains (mutagenesis with the use of ethyl methanesulfonate). However, both Novobiocin ($3.5\,\mu g/mL^{-1}$) and streptomycin ($150\,\mu g/mL^{-1}$) resulted in bacterial resistance to these antibiotics. What is more is that the authors have demonstrated that RSVF1 strain that became resistant to the phage remains sensitive to PlyG. In bacterial culture, application of lysin caused morphological changes of bacterial cells and ultimately led to cell lysis. Also, purified lysin encoded by the Tsamsa phage is suggested to be used in *B. anthracis* biocontrol due to its broad lytic spectrum that lysed more strains belonging to the *B. cereus* group than complete phage and which goes outside *B. anthracis* strains [56]. Inal suggested that in the case of anthrax infections lysin should be applied as soon as possible, before the lethal level of toxin is reached [32].

Porter et al. described PlyB lysin which showed lytic activity against a *B. anthracis*-like strain (ATCC 4342). The enzyme has muramidase activity, whereas PlyG is an amidase [107]. It is presumed that this lysin may be a new defensive tool in the face of bioterrorism danger. Lysins have some advantages over phages as the capsule is not an obstacle for PlyG to access the bacterial cell wall and may destroy encapsulated forms of bacilli. They show high specificity, not disturbing another bacterial species, and strong enzymatic activity; moreover the enzymes allow destruction of bacteria within seconds or minutes [108]. In *in vivo* experiments it was showed that PlyG applied in mice intraperitoneally did not cause evident toxic effects [86]. Another prevalence of these enzymes is that the resistance to them is induced rarely or not at all in comparison with whole phage particles. What is more is that it was observed that purified lysin isolated from Tsamsa phage was characterized by broader lytic activity than it was observed in the case of phage host range [56]. This phenomenon may be useful for biocontrol and decontamination not only in the case of *B. anthracis* threat but also in the instances of other *B. cereus* group bacteria contamination.

Sozhamannan et al. suggested that applying a combination of two different phages (γ and AP50c) with different lytic spectra may be a better alternative for therapy of anthrax, phage-based diagnostics, and disinfection of areas contaminated with anthrax bacilli [66]. Similarly, Inal stated that a phage cocktail (which has the ability to lyse most *B. anthracis* strains) should be prepared and tested as an optimal antianthrax agent [32]. Also, Porter et al. proposed feasible application of the combination of two different lysins, PlyB and PlyG, which exhibit different lytic activity and cleave different peptidoglycan bonds [107].

It was suggested that phages, especially a combination of different phages, may be used in a spray form applied to skin and clothes surface and into the respiratory tract [86].

5.2. B. anthracis Spore Decontamination. Although the use of phagesagainst *B. anthracis* is mainly limited to vegetative forms of the bacteria, there are phages that may be used for removing anthrax spores. Anthrax spores are not metabolically active, and they may be inactivated by physical methods (gamma irradiation, ultraviolet light, and high pressure) that are not safe for humans [71, 109]. There is a need to find a method of disinfection that is highly effective and safe. This form of *B. anthracis* is the most dangerous one as a potential terrorist bioweapon.

The spore cortex is protected by a proteinous coat against, for example, lysozyme. In the germinating state the porosity of coat is increased (even during 10 minutes of incubation in conditions inducing germination) [86]. Fu et al., usingcryoelectron tomography, describedthe structure of the SBP8a phage active against both vegetative and spore forms of *B. anthracis* and the molecular mechanism of phage infection

[110]. The phage showed the possibility to recognize and adhere to the surface of spores and eject its DNA inside the spore by the conformational changes of phage structures (at high SBP8a concentration, approximately 10^8 pfu/mL).

Application of phages (isolated from soil) in the aerosol form to germinated spores of the *B. anthracis* Sterne strain caused effective destruction of spores, but the effect was mainly observed when high titer lysate was applied (2.8×10^8 pfu/mL, 3.5×10^8 pfu/mL) [59]. The *B. anthracis* Sterne strain is a surrogate for virulent *B. anthracis*, which enables safe conduct of experiments on the avirulent *B. anthracis* Sterne strain and, according to data, the substitution does not significantly change or limit the results of the studies [71]. But using this strain guarantees safety—especially laboratory personnel who work on the *B. anthracis* are exposed to the risk of anthrax infection—and gives the possibility to conduct research on these dangerous bacteria.

As was observed, phages that are used against anthrax spores should be resistant to harsh environmental conditions, for example, dryness, ultraviolet radiation, extreme temperatures, and bodily fluids,to maintain ability to kill bacteria [71]. This feature would be important especially in the case of the disinfection application of spores (because of their high resistance to different factors).

6. Bacteriophages in Foodborne Pathogen Disinfection

There may exist the possibility to use other pathogens belonging to the *B. cereus* group in a bioterrorism attack. Bacteriophage typing may be useful for detecting food contamination with *B. cereus* [111], due to the fact that this method is cheap and convenient and seems to be fairly accurate [72]. The FDA approved the use of bacteriophages in order to guarantee food disinfection [112, 113]. There is a possibility and permission to apply bacteriophages providing food safety. To inhibit *B. cereus* contamination, the use of BCP78 phage isolated from fermented food was proposed [112].

Bacteriophages infecting *B. cereus* may be helpful in destroying this foodborne pathogen. For example, two phages, FWLBc1 and FWLBc2, which were isolated from soil, reduced the pathogen in mashed potato (by >6 $\log_{10}$ cfu/mL during 24 h). Because of the phages' narrow lytic activity, it has been suggested to use them as a component of phage cocktails [114]. This high specificity may constitute a disadvantage in using phages against foodborne pathogens, due to the complex composition of bacteria that contaminate food. A broad spectrum of inhibition of bacterial growth has been shown for Bc431v3 phage. It lysed bacteria belonging to the *B. cereus* group and *B. licheniformis*, *B. megaterium*, and *B. psychrosaccharolyticus*. The BPS10C and BPS13 phages that showed lytic activity against *B. cereus* were able to completely inhibit bacterial growth (bacteria belonging to the *B. cereus* group) for up to 6 h [115]. New phages with proven activity against *B. cereus* are still being isolated [116]. But it is extremely important that in their genomes phages do not encode genes responsible for lysogeny, toxin production, and genes affecting the pathogenicity of bacteria and antibiotic resistance [72]. The lack of them makes phage application safe for humans and increases phage application as a strategy of biocontrol of bacteria belonging to the *B. cereus* group.

Endolysins may be successfully applied in the case of *B. cereus* contamination [108]. For example, the LysB4 lysin isolated from the B4 bacteriophage was reported as the first endopeptidase among endolysins obtained from the *B. cereus* phages. Interestingly, the enzyme not only shows broad lytic activity against *B. cereus* strains but also lyses Gram-negative strains, for example, *E. coli* strains, in comparison with the phage lytic spectrum, which, most frequently, is limited to one *B. cereus* strain. This feature enables the enzyme to be an effective antibacterial agent active especially against foodborne pathogens. Furthermore, the lysin destroyed bacteria in merely 15 min and, according to Lee et al., this enzyme seems to be a perfect candidate as a biocontrol agent in the case of *B. cereus* contamination [61]. Endolysin BPS13 isolated from the BPS13 phage was highly temperature-stable; for example, it displayed lytic activity even at 100°C (suspended in glycerol) [115]. Yuan et al. isolated PlyBtSC33 endolysin from the *B. thuringiensis* BtSC33 phage [117]. The authors showed that this agent may be potentially used for disinfection purposes, had high temperature resistance, and showed a broad lytic spectrum (low lytic activity against *B. thuringiensis* but higher activity against *B. anthracis* and *B. cereus* strains). High thermostability may be useful in lysin application against food poisoning caused by *B. cereus*, especially in the heat treatment process. This endolysin may also be considered in anthrax treatment.

The lytic protein E33L that caused the lysis of *B. anthracis* was isolated from the genomeof *B. cereus* [118]. It was an N-acetylmuramoyl-L-alanine amidase, active against both *B. anthracis* and closely related strains belonging to the *B. cereus* group. This enzyme induced complete lysis already in nanomolar concentrations *in vitro* (almost 99% lysis of *B. anthracis* was achieved at 50 nM in 60 min). What is more is that the protein was active against *B. cereus* strains. An advantage of this agent is that the enzyme does not seem to be degradable by bacterial proteases, and furthermore it showed significantly higher lytic activity than *Bacillus*-phage-encoded endolysins.

The biothreat danger is a real possibility, and regardless of how the attack occurs (through water, air, mail, food contamination, soil, insects, and public transport) people should have a foolproof tool for rapid detection and identification and a possibility to treat patients from these dangerous (probably drug-resistant) pathogens. The control of *B. cereus* group bacteria, especially *B. anthracis*, is important in prevention and detection of bioterrorist attacks involving food contamination with regard to human health safety and economic reasons. We suggest that phages (whole particles or their purified endolysins) may constitute a good prospect in this area.

7. Concluding Remarks

The past two decades have proved that bioterrorism is a real threat which needs to be properly controlled. Recent

developments in phage therapy confirm that it may provide a reliable countermeasure preventing serious consequences of a terrorist attack using deadly bacteria, especially those resistant to antibiotics. Phage-mediated elimination of *Bacillus cereus* group bacteria, especially *B. anthracis*, seems to be an efficient tool against the potential use of such bacteria as a terrorist bioweapon.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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