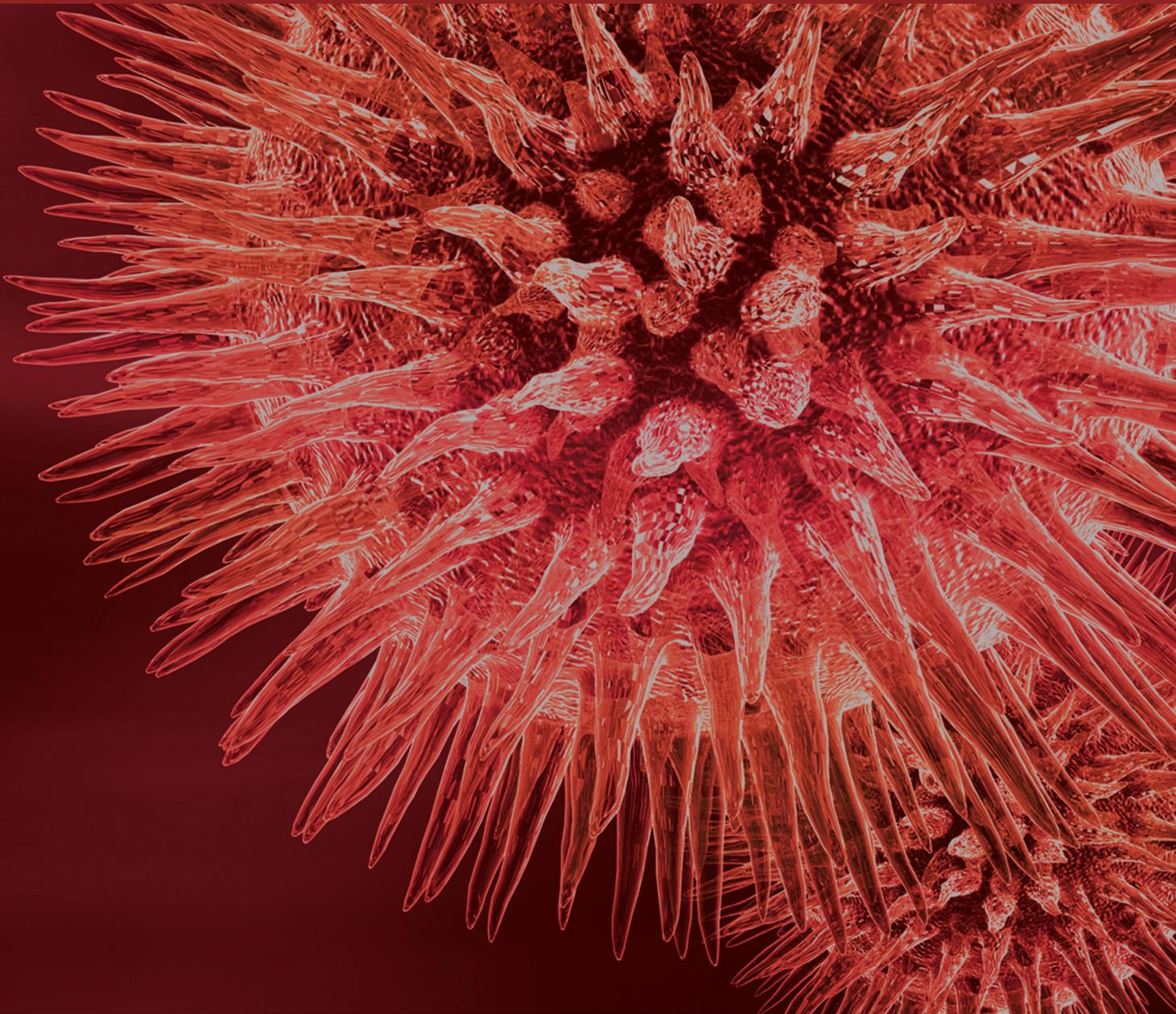


Emerging Microbial Concerns in Food Safety and New Control Measures

Guest Editors: Moreno Bondi, Patrizia Messi, Prakash M. Halami,
and Chrissanthy Papadopoulou





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Editorial

Emerging Microbial Concerns in Food Safety and New Control Measures

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Food-borne diseases are a widespread and growing public health and economic problem. Recent modifications in food production and processing practices and ever-changing food habits of the consumer are important factors for the incidence of food-borne infections. Many recognized pathogens are of great concern today and new challenges have appeared in the recent years as the role of the interaction between pathogens during infection.

Despite chill chains, chemical preservatives, and a better understanding of microorganisms, food-borne diseases (FBD) represent an important health problem for developed and developing countries. It has been estimated that about 30% of people in industrialized countries suffer from a food-borne disease each year (about 76 million cases of illness, 325,000 hospitalization cases, and as many as 5000 deaths in the United States annually [1]) and 25% of all foods produced globally are lost due to microbial spoilage. The European Food Safety Authority (EFSA) and the European Centers for Disease Control (ECDC), analyzing the information on the occurrence of zoonosis and FBD in 2010 submitted by 27 European Union Member States, reported a total of 5,262 food-borne outbreaks, causing 43,473 human cases, 4,695 hospitalization cases, and 25 deaths. Most of the reported outbreaks were caused by *Salmonella*, *Campylobacter*, bacterial toxins, and viruses. In particular, the scientific report of EFSA and ECDC 2012 [2] shows the decreasing trend in case numbers of a “classic” pathogen as *Salmonella* continues, whereas for other etiological agents of FBD a slight or no

decrease (*Listeria monocytogenes* and *Campylobacter*, resp.) or an increase (verotoxigenic *Escherichia coli*) in human cases has been observed. Another common cause of FBD worldwide is *Staphylococcus aureus*, responsible of an estimated 241,000 illnesses per year only in the United States [3].

In this special issue you will find an in-depth analysis on the importance of Staphylococcal food-borne disease. Recent findings of high prevalence of *S. aureus* including MRSA in raw retail meat impose a potential hazard to consumers, both as classic FBD and as a potential source of colonization of food handlers. An emerging problem that involves this field is therefore the increase of multidrug resistance in pathogenic, opportunistic, and spoilage bacteria, which can reach humans through the food chain. In recent years, for example, a new strain of *S. aureus*, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), has been recognized as a novel pathogen that has become a rapidly emerging cause of human infections [4]. As demonstrated by scientific evidence [5–7], genes for resistance may be transferred by mobile bacterial genetic elements frequently together with those encoding for virulence factors, a phenomenon that may lead to the appearance of microorganisms with increased pathogenicity.

This special issue also shows that, in some cases, the same pathogenic microorganisms, as well as representing a direct risk to health, can make the host more receptive to infection by other types of microorganisms, such as food-borne infections by protozoa. *Giardia duodenalis* is often seen

as an opportunistic pathogen and one of the major food and waterborne parasites. For example, the *Giardia* coinfection with pathogens like *Vibrio cholerae* and *Rotavirus* seems to play a significant role in the occurrence of this parasite. Some important factors are responsible for the regulation of the diarrheal disease spectrum of a population, as the age distribution of the diarrheal cases that was very much dependent on the coinfection rate of *Giardia* infection.

Outbreak investigations have suggested that a lot of risk factors linked to the raw ingredients, improper handling of cooked or processed food, an inadequate cleaning, and disinfection of equipment used in food processing, are the main source of contamination. For this reason, it is important to have new technologies that make it possible to determine such problem in real time. Here you can find a new method to detect food contamination by sensory analysis of foods. A novel electronic nose (EN) represents the tool that provides this capability. The characteristic flavour of volatile organic compounds (VOCs), the so-called fingerprint, may offer information about safety and quality of food, performing sometimes as an indicator of process mistakes as well [8]. Indeed some volatile compounds can originate from biochemical processes of food as a consequence of technological food chain or product storage. Unwanted smell, the so-called off-flavour, may involve substances originating from the metabolism of spoilage microorganisms, bacteria, and fungi that adulterate naturally or unintentionally the food before or during its production [9]. This EN could represent a rapid mean for controlling and improving the microbiological quality of food and a potential and useful tool for the early detection of microbial growth.

In this special issue a method to determine the actual risk of cereulide toxin production, by *Bacillus cereus*, in different types of food is also reported. Several *B. cereus* strains possess the genetic fittings to produce two different types of toxins, the heat-stable cereulide or different heat-labile proteins with enterotoxigenic potential. Unlike the diarrheal toxins, cereulide is (pre-) formed in food and can cause food-borne intoxication shortly after ingestion of contaminated food. It is possible to use a lux-based real-time monitoring system to assess the significance of the detection of emetic strains and to determine the actual risk of cereulide toxin production in different types of food. Luciferase signals were quantified with software and foods were categorized into three main classes regarding their toxin formation capability: high-risk, risk, and low risk foods. This generalized scheme allows a basic preevaluation of foods and their ingredients concerning their capability to support cereulide formation and should facilitate hazard identification.

Even if the use of refrigeration temperature is the most employed preservative method, spoilage and psychrotrophic pathogens can grow at low temperatures, representing a threatening public health concern and shortening the shelf life of raw foods. Many outbreaks associated with fresh ready to-eat produce have been previously reported due to *E. coli*, *Listeria*, and *Salmonella* [10]. If bacterial development could be delayed or inhibited, it would be possible to obtain a great advantage relatively to the public health and the shelf life of products. Actually, the consumer preferences are moving

towards foods containing lower levels of chemical preservatives, maintaining characteristics of fresh or natural products. Therefore, the preventive measures could be represented by sanitization methods alternative to the use of chemicals. A new microbial challenge in food safety, reported in this special issue, will be the use of physical treatment or the addition of natural preservatives endowed with antimicrobial capability. Recent studies demonstrate the bactericidal efficacy of alternative nonthermal light technologies and their potential as decontamination strategies in the food industry. A range of nonthermal technologies have already been successfully implemented on a number of ready-to-eat fruits and vegetables [11]. Exposure of microorganisms to visible light, particularly at wavelengths of 405 nm, has been shown to be effective in inactivating a range of Gram-positive and Gram-negative bacterial species [12]. Thus, these alternative nonthermal disinfection light techniques could find potential applications for decontamination in the food industry.

The other alternative to chemical food preservatives is the use of lactic acid bacteria (LAB). LAB strains are food-grade organisms that may be used in biopreservation strategies due to their ability to produce several antimicrobial compounds, including organic acids, hydrogen peroxide, and bacteriocins [13]. These microorganisms and their products could be employed as starter cultures [14] and natural preservatives [15] or could be entrapped in a polymeric film for its potential use in the active food packaging field [16]. The study reported in this special issue has provided the first genetic characterization of bioactive *Leuconostoc* spp. isolated from Algerian raw camel milk because of its beneficial effects on human health. *Leuconostoc* spp. plays a crucial role in food biopreservation through the production of bacteriocins with different inhibition spectra and exerts beneficial effects on the microbiological stability and production of aroma compounds in various food products. This study also represents the first report on the application of MALDI TOF MS analysis for the faster and more reliable identification of *L. mesenteroides* based on their low molecular-weight protein profile. The application of MALDI TOF peptide mass fingerprinting has been successfully applied to this bacterial group and has proven to be a simple, quick, and inexpensive complementary method for bacterial identification at the species level.

This editorial collects a brief summary of the topics discussed in the articles that are published in *Emerging Microbial Concerns in Food Safety and New Control Measures*. We hope that readers of this special issue will find some information of interest in order to expand their knowledge in this field and to increase their level of attention on matters here reported.

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Patrizia Messi
Prakash M. Halami
Chrissanthy Papadopoulou
Simona de Niederhausern

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Research Article

Association between *Giardia duodenalis* and Coinfection with Other Diarrhea-Causing Pathogens in India

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Giardia duodenalis, is often seen as an opportunistic pathogen and one of the major food and waterborne parasites. Some insights of *Giardia* infestation in a diarrhoea-prone population were investigated in the present study. Our primary goal was to understand the interaction of this parasite with other pathogens during infection and to determine some important factors regulating the diarrhoeal disease spectrum of a population. *Giardia* showed a steady rate of occurrence throughout the entire study period with a nonsignificant association with rainfall ($P > 0.05$). Interestingly coinfecting pathogens like *Vibrio cholerae* and rotavirus played a significant ($P \leq 0.001$) role in the occurrence of this parasite. Moreover, the age distribution of the diarrhoeal cases was very much dependent on the coinfection rate of *Giardia* infection. As per our findings, *Giardia* infection rate seems to play a vital role in regulation of the whole diarrhoeal disease spectrum in this endemic region.

1. Introduction

Giardia duodenalis is present worldwide but is more prevalent in developing countries where the lack of sanitation and hygiene awareness is a matter of concern [1, 2]. Considering its high endemicity in some countries, research on *Giardia* is of low priority as the infection it causes is self-limiting, a situation that enhances its propagation. Giardiasis is caused by the protozoan parasite *Giardia duodenalis* [3] which is usually transmitted through ingesting contaminated food and water. A wide variety of pathogens can cause diarrhea, but *G. duodenalis* impacts the economic growth of a country by affecting the Disability Adjusted Life Year (DALY) rates [4]. Giardiasis has much lower mortality rates associated with it than do other diarrheagenic pathogens such as *Vibrio cholerae* or *Shigella* [5]; nevertheless, it may still play an important role in regulating the spectrum of diarrheal diseases in diarrhea-prone regions. The study described herein was designed to survey the prevalence of *G. duodenalis*

among diarrheal patients within Kolkata, India. Kolkata is a densely populated city with a variable socioeconomic and climatic background and is frequently affected by outbreaks of diarrheal disease; hence that is why the area was chosen for disease transmission studies [6].

Fecal samples were tested from patients attending the Infectious Diseases and Beliaghata General (IDBG) Hospital in Kolkata city throughout a period of 56 months. These patients only complained of diarrhea. A systemic sampling procedure [7] allowed us to collect enough data to demarcate the catchment areas for diarrhea within the city and to interpret the epidemiological aspects of *Giardia* infestation in an urban region of this developing country.

2. Methods

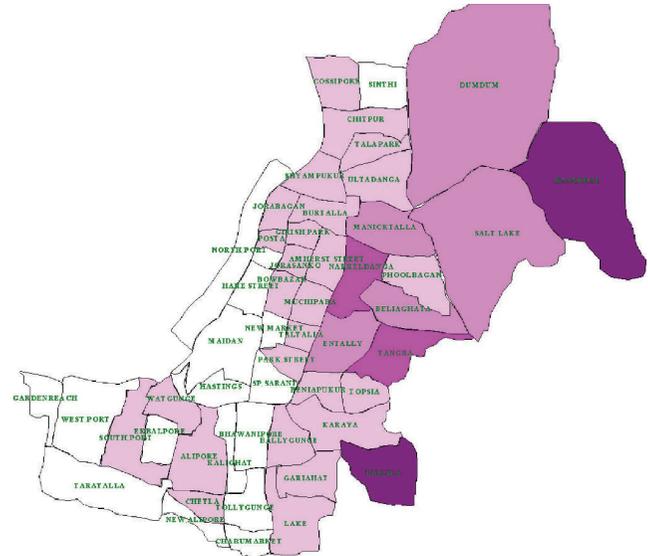
2.1. Ethics Statement. This study received ethical clearance from the National Institute of Cholera and Enteric Diseases (NICED) ethical committee, the host institute.

2.2. Study Design. The study was performed through collaboration between NICED and IDBG Hospital, Kolkata. IDBG is located within the city of Kolkata and is the largest infectious diseases hospital in India. IDBG treats around 25000 cases of diarrhea every year and most of these patients are residents of the city [6]. Thus, the prevalence of diarrheal diseases in the city can be estimated by surveying IDBG patients. Every fifth patient visiting IDBG who complained of only diarrheal symptoms on two randomly selected days per week was enrolled in the study. The study ran from November 2007 to June 2012. A single fecal sample was sent to the laboratory for analysis by trained healthcare professionals who also obtained the patient's background history via a systematically designed questionnaire. Patient consent for the study was obtained at the same time. The system remained unbiased with regard to sex, age, or other physical factors with nearly proportional distribution of male and female subjects and age ranging from 0 to 60 years in the majority of cases.

2.3. Screening for *G. duodenalis* in Stool Samples. *G. duodenalis* was detected in stool samples by using three different procedures. Stool samples were divided into three aliquots immediately after reaching the laboratory. The first aliquot was used for microscopic analysis with iodine wet-mount and trichrome staining [8] after concentration using "Ridley's concentration technique" [9]. The second aliquot was used in an antigen capture enzyme-linked immunoabsorbent assay using a GIARDIAII kit (TechLAB, Blacksburg, VA, USA) as per the manufacturer's protocol. DNA was extracted directly from the third aliquot of each stool sample using a DNA Stool Minikit (Qiagen, USA), according to the manufacturer's protocol. PCR was performed using *G. duodenalis*-specific primers and the DNA extracted by the kit as template following previously published protocols [7, 10]. All of the *G. duodenalis*-positive cases were also investigated for coinfections with other common pathogens as described previously [7]. The bacterial and viral coinfection status of a sample was investigated with assistance from Drs. T. Ramamurthy, T. Krishnan, and M. C. Sarkar in their laboratories at NICED [6].

2.4. Statistics and GIS Mapping. Data were entered into the predesigned format of the *pro forma* in the SQL server that has an inbuilt entry validation checking facilitated program by trained data entry professionals. Data were randomly checked and matched for consistency and validity. Edited data were exported and analyzed using SPSS.19.0 and Epi-info 3.5.4 [11].

The inferential age group was explored for *G. duodenalis*-positive cases by multinomial logistic regression [12, 13]. The aim of this was to determine the age groups that were most likely to be infected with *G. duodenalis*. Five age groups were classified, that is, up to 5 years, >5–10 years, >10–20 years, >20–30 years, 30–40 years, and >40 years, and were coded as 1–6, respectively. The relationships between the risk-dependent variable and each of the categorical explanatory variables are shown in Table 1. Infections caused by *G. duodenalis* were classified "1" when the pathogen was present



Giardia lamblia
 □ Missing or excluded ■ 22.01–33.00
 ■ 1.00–11.00 ■ 33.01+
 ■ 11.01–22.00

FIGURE 1: *Giardia duodenalis* distribution area. Map of the study region showing the catchment areas for the *Giardia duodenalis* cases according to our surveillance report (November 2007 to June 2008).

or "2" when absent. The extreme values of the classified age group were fixed as a reference category.

Associations between *G. duodenalis* infection and other variables such as rainfall or coinfection with other pathogens were tested using EpiInfo 3.5.4. Where the presence of *G. duodenalis* was considered an outcome variable, factors like rainfall, overall coinfection, and major coinfection were assigned as dependent variables. Where the *P* value was ≤ 0.05 , this was considered a valid association [14].

A choropleth map was constructed to display the data from the area where all the positive samples had originated within the city [15]. For this map, the different colors and patterns were combined to depict the different values of the attribute variable associated with each area. Each area is colored according to the category into which its corresponding attribute value had fallen. *G. duodenalis*-positive cases were embedded on the thematic map by the geographical information system (GIS) to visualize the infections. The boundary map shows that the prevalence of *G. duodenalis* was highest in Rajarhat and Tiljala (31.0%), followed by Narkeldanga and Tangra (22–33%), while the values for Dum Dum, Salt Lake, Beliaghata, Maniktala, and Entally regions ranged from 11 to 22 percent (Figure 1).

3. Results and Discussion

Single stool samples from 4039 diarrheal patients were examined throughout a 56-month period, and 413 (i.e., 10.2%) of them tested positive for *G. duodenalis*. All the data were categorized on a monthly basis to assess any

TABLE 1: Association between rainfall and *Giardia* prevalence: average seasonal rainfall in the study region (Indian Meteorological Department Database), average *Giardia* detection rates, and the percentage of Giardiasis among all diarrheal cases.

Season	Average rain (mm)	Monthly average <i>G. duodenalis</i> -positive cases	Total diarrhea cases	Monthly average <i>G. duodenalis</i> -positive (%)
Premonsoon/summer 08	153.4	11	73	15.05
Monsoon 08	1291.7	12.75	103.5	12.02
Postmonsoon 08	70.3	12	110.3	10.1
Winter 09	3.4	4.5	91	4.8
Premonsoon/summer 09	251.8	11.7	123	9.26
Monsoon 09	971.5	18.75	141	13.5
Postmonsoon 09	95.7	5.7	73.3	7.73
Winter 10	16.6	2	34	6.3
Premonsoon/summer 10	143.7	7.3	67	10.83
Monsoon 10	787.4	4	48.25	8.32
Postmonsoon 10	138.8	4.7	48	10.3
Winter 11	5.4	4	37.5	10.7
Premonsoon/summer 11	245.2	5	51.7	10.03
Monsoon 11	1391.6	1.75	35.5	4.87
Postmonsoon 11	29.5	2.7	32	9.6

possible seasonality in *Giardia* prevalence. The percentage of *G. duodenalis*-positive cases detected was similar over the entire period with an average detection rate of around 10% each month (Figure 2(a)) and showed a significant correlation with the total number of diarrheal cases in each month ($P < 0.001$). It was evident that the total number of diarrhea cases decreased significantly towards the end of the survey, a trend similar to that observed with *Giardia*-positive cases (Figures 2(a) and 2(b)). *G. duodenalis* showed a statistically significant seasonality and strong association with the total number of diarrheal cases ($P = 0.001$); however, no significant association was found between the numbers of *Giardia*-positive cases and rainfall in the region ($P > 0.05$) (see Supplementary File 1 available online at <http://dx.doi.org/10.1155/2014/786480>) (Table 1). The number of *Giardia* cases increased during the midsummer to monsoon season (i.e., from May to August). Seventy-four percent of the *Giardia*-positive cases were found to be coinfecting with other pathogens, while the remainders were single infections. As per the literature, *Giardia duodenalis* infection may not be associated with diarrhea or related diseases in some cases and rather remain asymptomatic for a long period of time [16, 17], but twenty-six percent of sole infection in the diarrhea patient among the study population demonstrates the symptomatic nature of *Giardia* in this case. Coinfection with *Vibrio cholerae* was the most common (32%), followed by rotavirus (19%) (Figure 3(a)). As all the tests for *Giardia* and other pathogens were conducted over the same set of samples, so the chance of generating data artifact was minimized and the multiple infection could be considered as true coinfection. Infection with *Giardia* showed a strong positive relationship with the presence of other diarrhea-causing pathogens ($P < 0.001$) (Figure 3(b)). *Giardia* infection was very common in the lower age groups and statistically significant associations

TABLE 2: Multinomial logistic regression models exploring the significant predominant risk age group for *Giardia duodenalis* infection at IDBG, Kolkata (November 2007–July 2012).

Age in years	<i>Giardia duodenalis</i>	B	OR (95% CI)	P value
≤5 years (n = 960)	144	0.56	1.74 (1.29–2.35)	<0.001*
>5–10 years (n = 126)	35	1.33	3.79 (2.40–6.00)	<0.001*
>10–20 years (n = 375)	60	0.63	1.88 (1.30–2.71)	0.001*
>20–30 (n = 551)	64	0.26	1.29 (0.91–1.85)	0.150
>30–40 (n = 416)	37	−0.04	0.96 (0.64–1.46)	0.863
>40 years (n = 794)	73		Reference category	

n = sample number.
*Statistically significant.

were found for children ≤5 years and >5–10 years ($P < 0.001$) (Table 2). An age-dependent infection status was also apparent with the two major coinfecting pathogens, *V. cholerae* in the ≤5-year ($P < 0.001$) and rotavirus in >5–10-year ($P < 0.001$) group. Interestingly, coinfections of *Giardia* and other diarrhea-causing pathogens showed a marked decline with increasing age compared with infections with *Giardia* alone (Figure 4(a)).

In spite of observing a trend in the monthly isolation rate for *G. duodenalis*, no seasonality pattern could be inferred from the data; this may be because isolation of the parasite is dependent on the total number of diarrheal cases and this

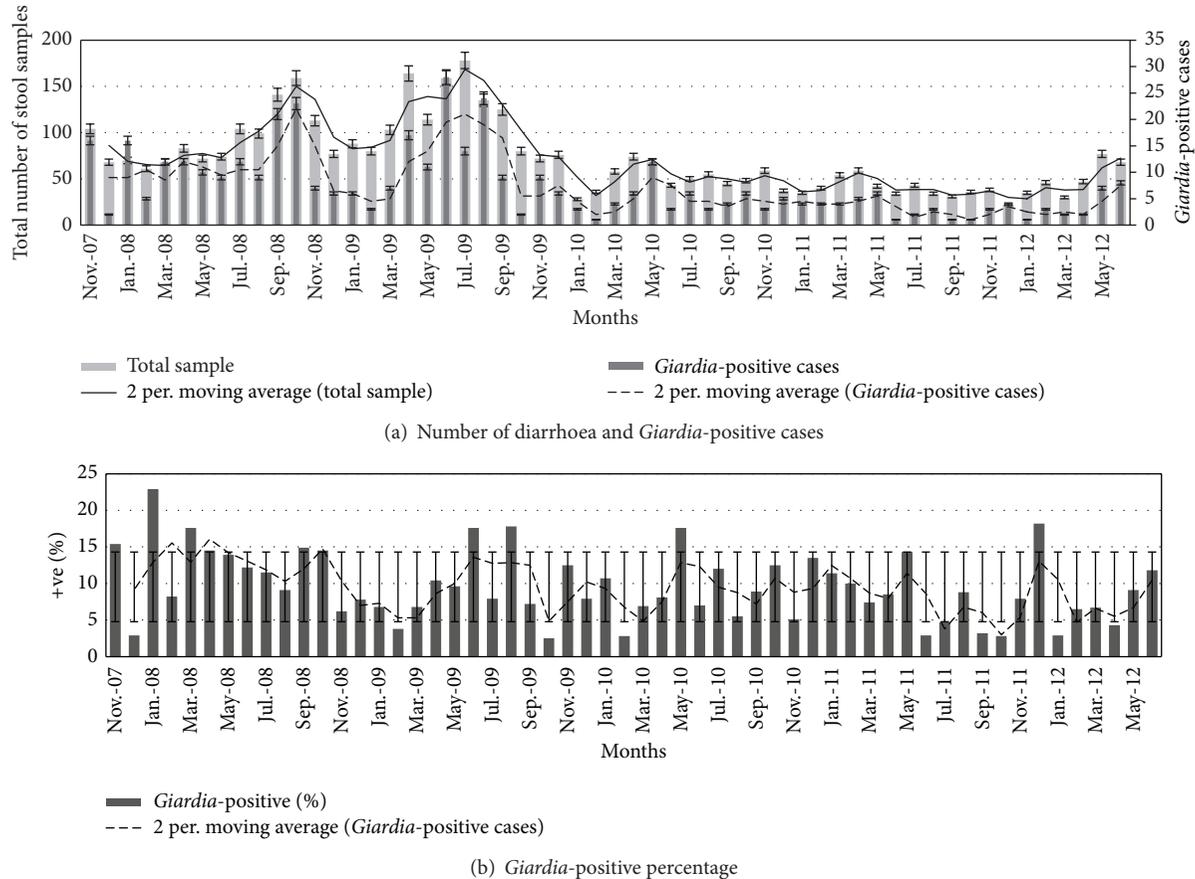


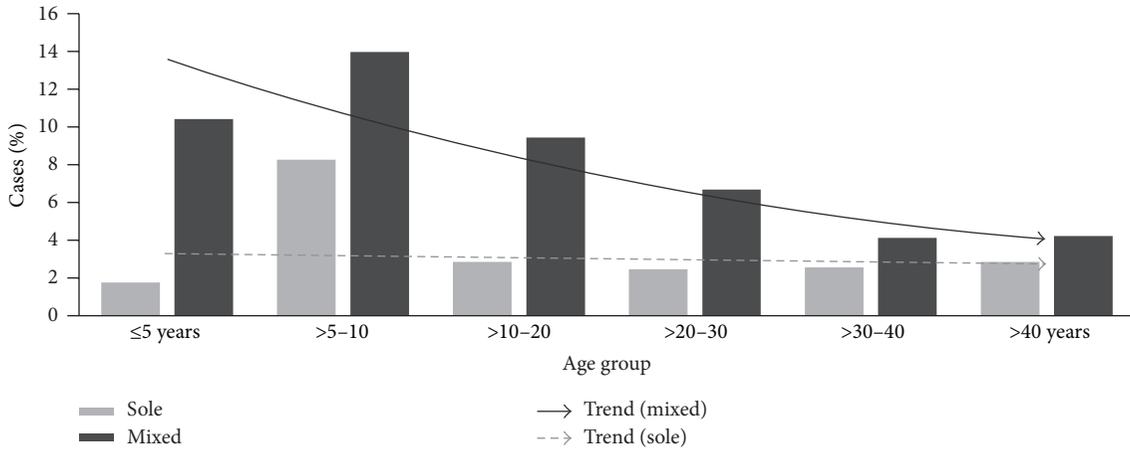
FIGURE 2: Month-wise distribution of diarrhoeal cases and *Giardia*-positive cases. (a) The total number of stool samples tested per month throughout the study period and the number of *Giardia*-positive cases recorded for the same period. Note that different y-axis scales have been used for clarity. Trend lines representing the sample distribution and *Giardia*-positive cases are similar. (b) Percentage of *Giardia*-positive cases each month. The data show constant deviation and a nonexplanatory trend line.

number changes according to the season. However, the steady rates of infection seen in the dry seasons could indicate that *G. duodenalis* is not dependent on rainfall. In this regard, the finding that *Giardia* infections were strongly associated with coinfection ($P < 0.001$) suggests that the parasite derives some advantage from the presence of other diarrhea-causing pathogens in the host, or vice versa. Similarly, *G. duodenalis* was found to be most prevalent in ≤ 5 -year and > 5 -10-year olds, suggesting that age can be a determining factor for increased susceptibility to Giardiasis. Interestingly, in both of these age groups, coinfections of *Giardia* and rotavirus in children ≤ 5 years and *Vibrio cholerae* in children above 5-10 years were common (Figure 4(b)). As with previous studies, infection with *V. cholerae* or rotavirus is common in the lower age groups [18] in the study region. This suggests that *Giardia* could in some way take benefit from the major pathogens prevalent in a particular population at a particular time. This could explain the lack of seasonality and steady infection rates among diarrhoeal cases in regions where *Giardia* is endemic. In the present study, the *G. duodenalis* infection rate is high in the monsoon or postmonsoon period, as did *V. cholerae* and other bacterial pathogens that are associated with water contamination from uncontrolled sewage dispersal in the

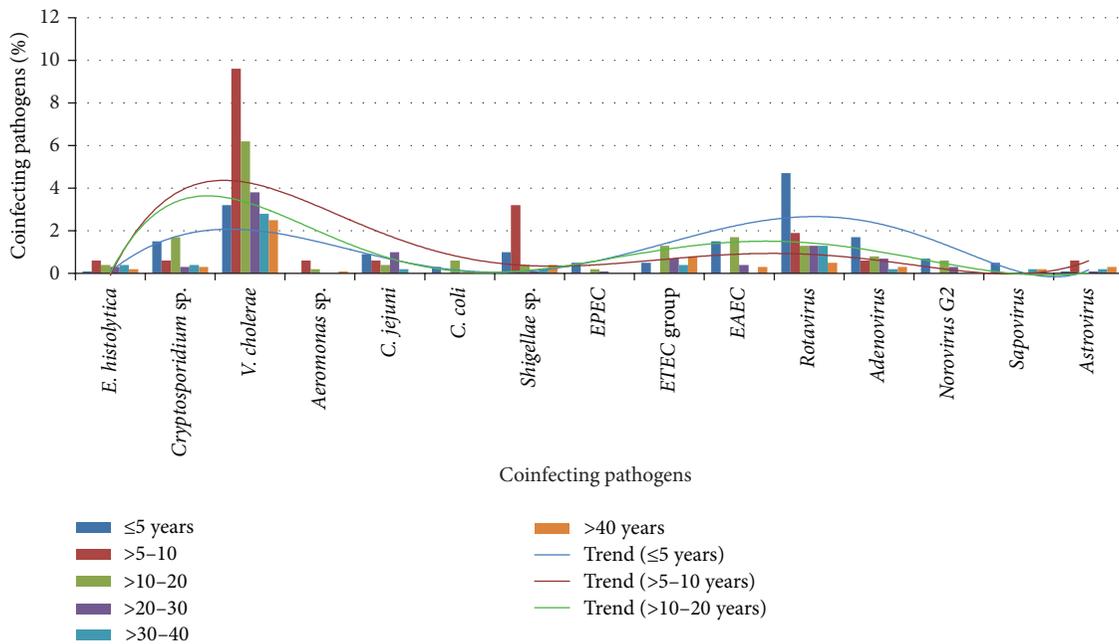
rainy seasons. However, the rate is also high in the winter, along with coinfecting pathogens such as rotavirus.

4. Conclusions

The high rate of *Giardia* infection seen throughout the study period across all climatic conditions and the significant association of *Giardia* with other major pathogens suggest that the parasite may play a role in regulating the spectrum of diarrhoeal disease in the study area. A statistically significant association with *Vibrio cholerae* and rotavirus across two different seasons suggests that *Giardia* may have evolved to survive in the diarrhoea-prone endemic region investigated herein. The opportunistic nature of *Giardia* is previously considered as an opportunistic pathogen so it can be a major reason for the observation. Otherwise, the coinfection status could be a reason for coexistence of *Giardia* and other pathogens in the infection source, that is, food and water. *Giardia* appears to be maintaining the characteristics of an ideal opportunistic pathogen, resulting in a steady but high prevalence rate in a population and eventually making the population more susceptible to other major diarrhoeal infections.



(a) Age distribution of sole and mixed *Giardia* infection



(b) Coinfection status of *G. duodenalis* stratified by age group

FIGURE 4: Age-wise distribution of *Giardia* and its relationship with coinfecting pathogens. (a) Distribution of single infections of *Giardia* and mixed infections with other pathogens across six age categories. Note the decreasing slope of the trend line in the older age groups. (b) Age distribution of *Giardia* cases according to their coinfection status with other pathogens. Trend line of ≤5 years shows that coinfection is highest for rotavirus, followed by *Vibrio cholerae*, and for the >5-10 and >10-20 age groups coinfection with *V. cholerae* is higher.

final analysis, evaluated all of the results, checked the paper, and gave final approval for paper publication.

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Research Article

Emetic *Bacillus cereus* Are More Volatile Than Thought: Recent Foodborne Outbreaks and Prevalence Studies in Bavaria (2007–2013)

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Several *Bacillus cereus* strains possess the genetic fittings to produce two different types of toxins, the heat-stable cereulide or different heat-labile proteins with enterotoxigenic potential. Unlike the diarrheal toxins, cereulide is (pre-)formed in food and can cause foodborne intoxications shortly after ingestion of contaminated food. Based on the widely self-limiting character of cereulide intoxications and rarely performed differential diagnostic in routine laboratories, the real incidence is largely unknown. Therefore, during a 7-year period about 4.300 food samples linked to foodborne illness with a preliminary report of vomiting as well as food analysed in the context of monitoring programs were investigated to determine the prevalence of emetic *B. cereus* in food environments. In addition, a *lux*-based real-time monitoring system was employed to assess the significance of the detection of emetic strains in different food matrices and to determine the actual risk of cereulide toxin production in different types of food. This comprehensive study showed that emetic strains are much more volatile than previously thought. Our survey highlights the importance and need of novel strategies to move from the currently taxonomic-driven diagnostic to more risk orientated diagnostics to improve food and consumer safety.

1. Introduction

Cereulide, an emesis-inducing toxin produced by a fairly homogenous group of *B. cereus* strains called “emetic *B. cereus*,” is a small heat-stable cyclic peptide [1]. The emetic poisoning caused by cereulide is usually characterized by vomiting starting after 0.5 hour to six hours after consumption of the contaminated food. Intoxications proceed mostly with mild symptoms and last normally not more than one day, but severe cases requiring hospitalization are increasingly reported (for review see Ehling-Schulz et al., 2004 [2], and Ehling-Schulz et al., 2011 [3]).

Because of the short period of illness, the emetic syndrome caused by *B. cereus* is presumably underreported

[4]. In addition, the symptoms of an emetic intoxication caused by *B. cereus* parallel the symptoms caused by *S. aureus* enterotoxins, bearing the risk of misdiagnosis of the disease. In the year 2011 the European Food Safety Authority (EFSA) reported an increase of 122.2% in the number of foodborne intoxications and toxicoinfections caused by *B. cereus* in Europe. The overall reporting rate was 0.04 cases per 100.000 inhabitants [5]. Even if intoxication with the emetic toxin cereulide in most cases produces only mild symptoms, consistently also fatal cases are reported [6–8].

Although *B. cereus* is an ubiquitous spore former, emetic strains are rarely found in the environment and their natural niches and entrance points into the food production and processing are largely unknown [4]. So far, mostly high-carb

food matrices, such as rice and pasta, as well as milk and dairy products, have been investigated for the presence of emetic strains of *B. cereus* [9–13], whereas other food matrices have rarely been included in the analyses. To improve HACCP-based concepts and prevent foodborne intoxications caused by emetic *B. cereus*, information on the general prevalence of emetic strains in foods of different origin is of utmost importance and data on the risk of toxin formation in different food categories are required.

This study therefore aimed to (i) investigate the prevalence of emetic *B. cereus* strains in a wide range of food matrices, covering foods from plant as well as animal origin, to identify potential contamination sources, and to (ii) facilitate hazard identification by exploring the potential of diverse food matrices for the risk of cereulide toxin production. In this context, a perennial survey from 2007 to 2013 was carried out, including food samples connected to emesis-related foodborne illnesses as well as samples not related to foodborne outbreaks. By using an *in situ* bioassay indicative of cereulide production levels, a general scheme for categorizing foods with respect to their risk of cereulide production was generated.

2. Material and Methods

2.1. Sample Material. Between the years 2007 and 2013 3,564 food samples from Bavaria were analysed for the presence of emetic *B. cereus* strains in the context of foodborne illness or outbreaks where the consumers showed symptoms of vomiting. The majority of samples were taken from the household of the diseased consumers and from restaurants, canteens, and catering companies. Additionally, the presence of emetic *B. cereus* strains in different food matrices ($n = 742$) was investigated in the scope of different monitoring programs. Food categories for the monitoring were chosen from both food of animal origin and food of plant origin. All samples were examined before their expiry date.

2.2. Microbiological Detection of *B. cereus* and Identification of the Cereulide Synthetase Gene *ces*. Emetic *B. cereus* strains were detected with qualitative and quantitative methods (for details see Ehling-Schulz et al., 2011 [3]). The qualitative detection was done weighting 10 g of sample material into 90 mL of tryptone-peptone-glucose-yeast (TPGY) broth and incubating at 30°C under aerobic conditions. After 24 h of cultivation 1 mL of the enrichment broth was taken for the molecular detection of the *ces* genes, which encode the nonribosomal synthetase responsible for the production of the peptide toxin cereulide. For detection of *ces*, a previously described probe-based diagnostic real-time-PCR assay was used [9, 15].

The quantitative detection of presumptive *B. cereus* was carried out using standard reference culture methods recommended by the International Organisation of Standardization (ISO) and the U.S. Food and Drug Administration (FDA). Samples were investigated using spiral plate count method on the Mossel agar [16, 17] or with a 3-tube 3-dilution most probable number (MPN) method [18–21]. Presumptive *B.*

cereus colonies were further differentiated by the detection of the *ces* gene either by real-time-PCR as described above or by using a conventional PCR system according to Ehling-Schulz et al., 2004 [22]. Depending on the results of these reactions the number of colony-forming units per gram (cfu/g) or MPN of emetic *B. cereus* cells per gram of sample was calculated following the standard methods recommended by FDA and ISO [16, 17, 21].

2.3. Bioassay-Based Risk Categorization of Foods. Analysis of the potential of food matrices to support cereulide production was performed by artificial contamination of 30 g portions with the bioluminescent *B. cereus lux* reporter strain F4810/72(pMDX[P₁/luxABCDE]) and an IVIS camera system as described earlier [23, 24]. Foods were provided by diverse manufacturers or were obtained from local consumer markets. In the case of powders and freeze-dried products (e.g., infant formulas and instant potato powder) or raw materials (e.g., rice and pasta) foods were prepared according to the manufacturers' instructions thereby simulating common household conditions. The contents of preportioned packaging units (e.g., single-sliced cheese or biscuit snacks) were combined and blended for 3 min with a stomacher to obtain homogenous testing matrices. Dry foods, such as dates, apricots, cocoa powder, and herbal salt, were additionally soaked with sterile water or pasteurized milk (1.5% fat content) as indicated. Matrices were filled into Petri dishes and inoculated to a final reporter strain cell count of 10³ CFU per gram. After an incubation step for 24 hours at 24°C, the luciferase signal intensities were quantified with a photon-counting intensified-charge-coupled-device (ICCD) camera (model 2400-32; Hamamatsu Photonics) and are shown as false-color renderings that were superimposed on gray-scale images of the respective food sample.

3. Results and Discussion

This study was designed to get a comprehensive overview of the prevalence of emetic *B. cereus* strains in both food samples from supposed foodborne intoxications and food samples from general food monitoring programs. These data should provide a profound basis for a better risk assessment concerning the emetic syndrome caused by cereulide producing emetic *B. cereus* strains. In addition, the influence of food matrix properties on cereulide production was evaluated using a previously established *lux* reporter system [24].

3.1. Prevalence of Emetic *B. cereus* in Foods Linked to Food-Borne Intoxications and in Nonfood Intoxication Associated Food Samples. Because most studies hitherto targeted only a very limited range of food matrices, such as rice and pasta (e.g., [25–27]), and samples were collected from very specific sites or during very short sampling periods (see e.g., [13]), prevalence data covering samples from different years and diverse food matrices are still missing. However, in the context of preventive consumer protection policy and for a comprehensive risk assessment, data about the prevalence of emetic strains in different food categories from a perennial

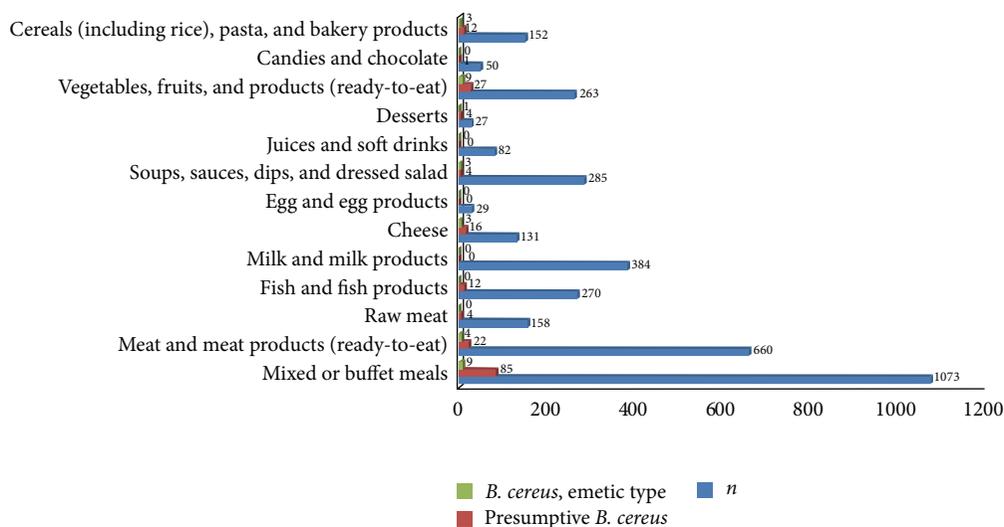


FIGURE 1: Presumptive *Bacillus cereus* and emetic strains in different food matrices investigated in the context of supposed foodborne intoxications.

sampling period are required. We therefore analysed 3.654 food samples obtained from suspected foodborne illness with a preliminary report of vomiting shortly (within a period from thirty minutes to six hours) after consumption of the suspected meal over a period of 7 years (2007 to 2013). The analysed samples covered a broad variety of food categories (Figure 1). Presumptive *B. cereus* was detected in 187 samples (5%) and emetic *B. cereus* strains were detected in 32 samples (1%). Interestingly, emetic strains were not only detected in farinaceous foods commonly linked to cereulide intoxication (e.g., [1, 6, 8]) but also in vegetables, fruit products, sauces, soups, and salads as well as in cheese and meat products (Figure 1). Recently, Doménech-Sánchez et al. [28] reported on an emetic outbreak linked to the consumption of tuna fish. These results emphasize that more data on the prevalence of emetic *B. cereus* in different types of foods are needed to decipher potential contamination sources.

In most samples tested positive for *B. cereus*, which have been analysed in the context of foodborne intoxications, emetic strains were found in levels $\leq 10^2$ cfu/g food matrix (see Table 1). All of these samples were tested negative for the presence of other foodborne pathogens, including *S. aureus* and its enterotoxins (data not shown). It is therefore assumed that *B. cereus* was indeed the etiological agent of the reported outbreaks. The detection of emetic *B. cereus* in low levels in samples from suspected foodborne illness could be an indication that the bacteria themselves were reduced by the food production and processing procedure, but the preformed heat- and acid-stable toxin cereulide was not eliminated or inactivated. In addition, it is known that the capability of toxin formation varies significantly among emetic *B. cereus* strains and the actual toxin production depends on external parameters [11, 23, 29, 30]. These examples highlight the need of novel diagnostic strategies, moving from taxonomy to more risk orientated differential diagnostics (for review see Ehling-Schulz and Messelh usser, 2013 [31]).

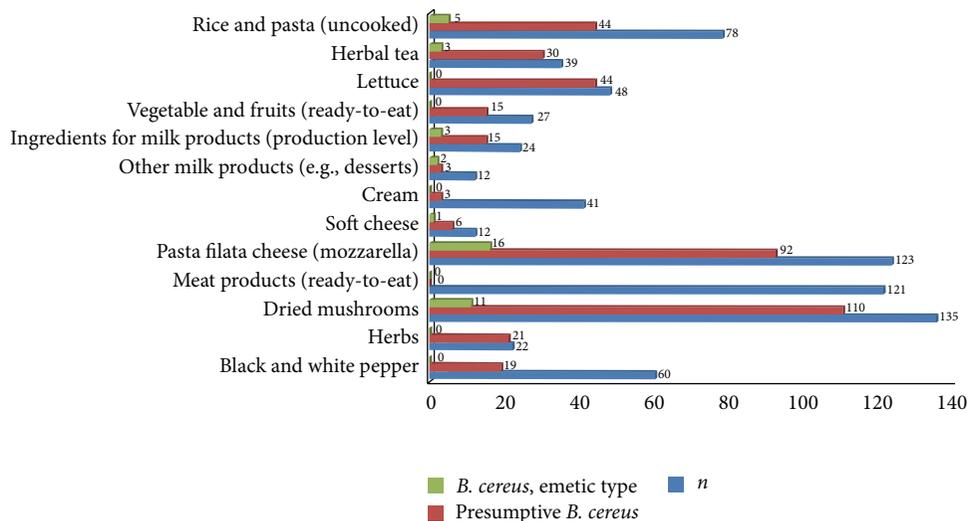
To gain a deeper insight into food associated natural niches of emetic *B. cereus* and potential contamination sources, 742 food samples of animal and plant origin were investigated for the presence of emetic *B. cereus* strains within different monitoring programs (Figure 2). For food of animal origin, samples were grouped in categories that have been reported in the context of foodborne illness, for example, ready-to-eat meat products, cheese, and cream. For food of plant origin, food matrices were investigated that could be possible contamination sources for ready-to-eat food, such as herbs, spices, and dried mushrooms or fresh foods, such as lettuce, fruits, and vegetables. Emetic strains were most frequently found in pasta filata cheese obtained from retail level (13%), in dried mushrooms (8%), and in herbal teas (8%). The detection rates in these matrices were even higher than in uncooked rice and pasta (6%), whereof also 78 samples were investigated. Overall, 10% of presumptive *B. cereus* strains, isolated in the context of monitoring programs, possess the *ces* gene and therefore the ability to produce cereulide toxin. These prevalence rates are slightly higher than the ones reported from previous studies (e.g., [25, 27]). One explanation might be that emetic *B. cereus* strains are easily overlooked in routine diagnostic since they frequently show an atypical phenotype and might, in addition, be outcompeted on nonselective agar media often used in microbial diagnostics [32]. The food category investigated could also significantly influence the percentage of emetic isolates detected. For instance, as our study showed (in food categories for which more than 50 samples were investigated) the percentage of emetic strains isolated from different food matrices varied between 10% (dried mushrooms) and 17% (pasta filata cheese) (see Figure 2).

However, not only the presence of strains but also the potential of food matrices to support cereulide synthesis should be considered for an accurate risk assessment, since unavoidable low-level contaminations with the spore formers might lead to intoxications or even large-scale outbreaks in

TABLE 1: Examples for potentially foodborne diseases caused by emetic *B. cereus* in Bavaria between the years 2007 and 2013.

Year	Diseased persons	Place	Food matrix	Level of emetic <i>B. cereus</i> (cfu/g)
2007	Several students after a cooking lesson at school	School kitchen	Hard cheese	<100 (only positive using a qualitative detection method, but detection of 2 μg cereulid/g*)
2007	One adult	Restaurant	Cooked pasta	3.8×10^5
2008	Several students	School canteen	Paprika filled with meat and rice	<100 (only positive using a qualitative detection method)
2009	One adult	Household	Cooked potatoes	<100 (only positive using a qualitative detection method)
2010	One adult	Restaurant	Cooked pasta with oysters	<100 (only positive using a qualitative detection method)
2010	Several adults	Canteen	Poulard breast in tomato sauce	<100 (only positive using a qualitative detection method)
2010	Several adults	Catering	Chana masala (cooked chickpea) with baked potatoes in curry sauce and cooked rice	Cooked rice: 2.8×10^4 (1 μg cereulid/g*) Cooked chickpea: <10 (only positive using a qualitative detection method, but detection of 0.3 μg cereulid/g*); see also Ehling-Schulz and Messelhaeusser, 2012 [14]
2011	Several children (1 to 3 years old)	Nursery school	Cooked pasta with tomato sauce	6.8×10^6
2011	Two adults	Restaurants	Cooked pork meat with potatoes	1.0×10^2
2011	One adult	Household	Cured and smoked meat	1.0×10^2
2012	Several students	Canteen	Raspberry quark	1.4×10^2
2012	One adult	Household	cooked mushrooms	1.9×10^7
2013	Several adults	Catering at a wedding	Vitello tonnato	6.1×10^7

* Currently, no officially validated method for the quantitative detection of cereulide in food matrices is available; therefore quantitative data on cereulide toxin are only shown for selected samples. However, recently a European initiative has been started to establish appropriate ISO methods (CEN/TC 275/WG 6).

FIGURE 2: Presumptive and emetic *B. cereus* in different food matrices investigated in the context of monitoring programs.

cases of improper storage and handling of prepared meals. Previous work showed that the risk of cereulide production is strongly connected with external parameters and varies significantly among different types of model foods that have been investigated so far [23, 24, 30].

3.2. *Broad-Scale Risk Categorization of Food Matrices concerning Cereulide Synthesis.* Although the EFSA stressed the necessity of identifying categories of foods that may pose a risk for human health with respect to cereulide contamination [4], a comprehensive evaluation of food matrices

TABLE 2: Bioassay-based categorization of 70 retail foods according to their potential for supporting cereulide production. Bioluminescence intensity produced by the cereulide synthesis reporter strain F4810/72(pMDX[P₁/luxABCDE]) was measured after 24 hours of incubation at 24°C. Representative images are shown in Figure 3 and Figures S1–S3. Threshold values established for risk categorization are listed in Table S1.

Low-risk foods	Risk foods	High-risk foods
Dried apricots	Reconstituted milk powder (organic)	Cereal-based reconstituted infant food (fruit flavour)
Dried apricots rehydrated with water	Dried dates rehydrated with water	Cereal-based reconstituted infant food
Infant food with yoghurt and fruits	Cheese slices with suisse flavour	Cereal-based reconstituted infant food (whole grain/apple flavour)
Crème fraîche	Cheese slices with mozzarella flavour	Dessert creme with cream/coffee flavour
Crème fraîche with herbs	Minced pork	Dessert creme with caramel flavour
Dried dates	Minced veal	Diet drink with vanilla flavour
Diet chocolate with cream filling	Cocoa powder with milk	Muesli with water
Cottage cheese (whole fat content)	Herbal salt (1% in water)	Muesli with milk
Fresh cheese (natural)	Latte macchiato drink	Semolina pudding (natural)
Fresh cheese with herbs	Camembert cheese (60% fat content)	Semolina pudding (vanilla flavour)
Fresh cheese with chilli flavour	Chocolate mousse	Semolina pudding (cinnamon flavour)
Yoghurt of fresh cheese with fruits	Pasteurized milk (1.5% fat content)	Boiled Jasmin rice (organic grains)
Yoghurt of fresh cheese with vanilla/fruit	Pasteurized cream (30% fat content)	Boiled Jasmin rice (parboiled grains)
Yoghurt of fresh cheese with raspberry	Chocolate biscuit with milk cream filling	Mashed potatoes (powder reconstituted with water)
Cocoa powder reconstituted with water		Mashed potatoes (made from cooked potatoes)
Curd cheese		Reconstituted skim milk powder
Curd cheese with vanilla flavour		Milk drink with nut flavour
Whey drink peach flavour		Rice pudding (natural)
Whey drink cherry/banana flavour		Rice pudding (strawberry flavour)
Nougat creme		Rice pudding (chocolate flavour)
Sauce carbonara		Rice pudding (vanilla flavour)
Chocolate bar with milk/caramel filling		Rice pudding (cinnamon flavour)
Soy bean sprouts		Boiled whole grain rice
Quark		Scrambled egg
Herbal salt (10% in water)		Soy milk
		Soy milk-based dessert with caramel flavour
		Soy milk-based dessert with vanilla flavour
		Reconstituted whole milk powder
		Mousse au vanilla
		Pudding with vanilla flavour
		Vanilla sauce

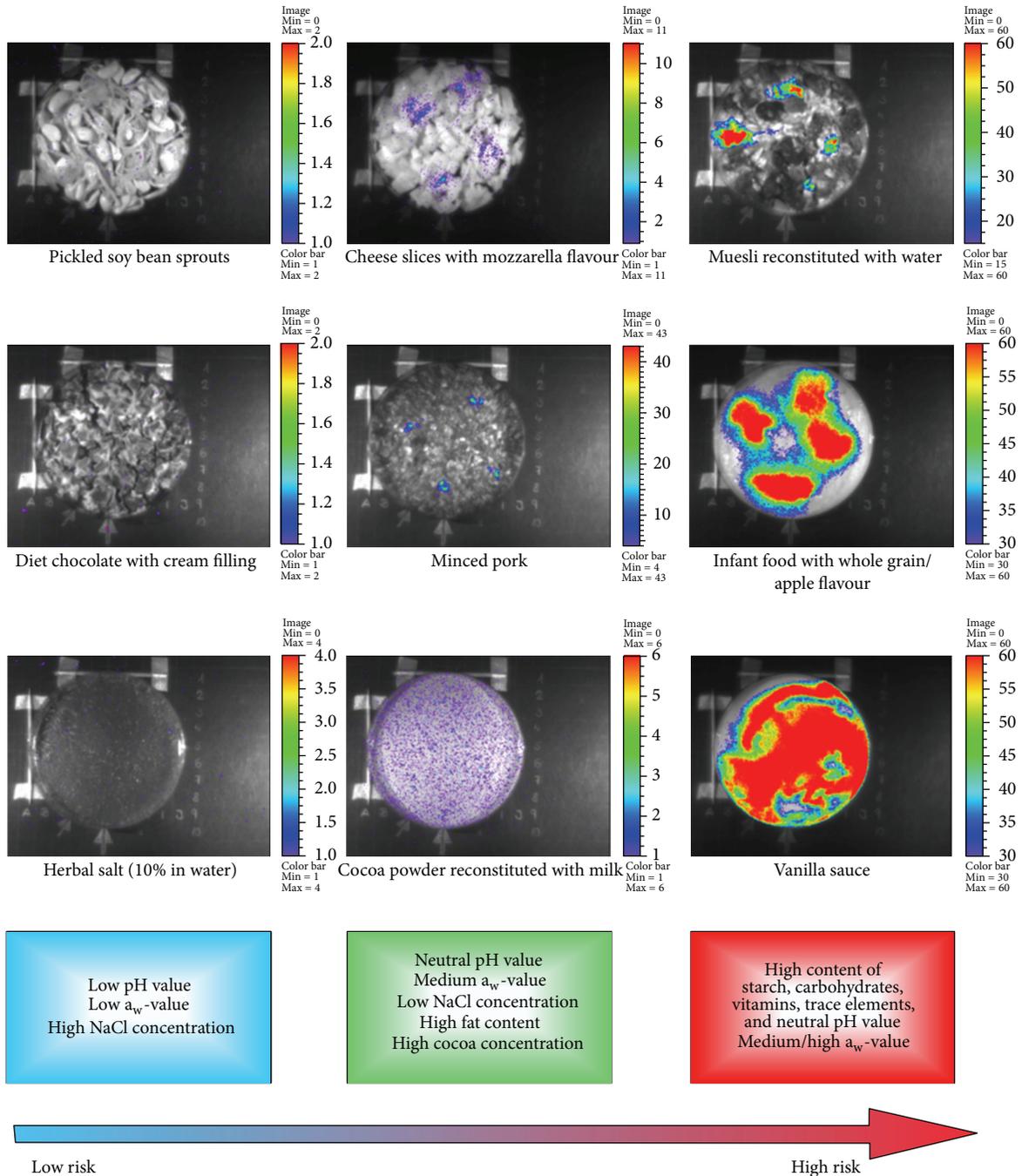


FIGURE 3: Scheme for abiotic factors influencing the activity of the *ces* NRPS promoter driving the synthesis of cereulide. The parameters were deduced from the examination of 70 foods and food ingredient using an emetic *lux* reporter strain [10]. The arrow denotes an increasing toxin formation capability with respect to the food composition. Examples of typical food matrices for each category are shown.

was hampered due to laborious, time-consuming, and error-prone methods to quantify cereulide amounts in foodstuffs. Recently, a SIDA-based method allowing the quantitative detection of cereulide has been developed [33]. However, alternative high-throughput methods to estimate the risk of toxin production in diverse food matrices are needed. The *lux*-based reporter system for real-time monitoring of toxin gene expression described by Dommel et al. [24] might represent an interesting tool in the latter context. We previously

showed that cereulide production in model food matrices is proportional to the intensity of the bioluminescence signals emitted by the engineered *B. cereus* reporter strain [23, 24].

In this study, we employed the *lux* reporter system for the analysis of a total of 70 retail products in order to decipher abiotic and nutritional factors, either promoting or suppressing toxin synthesis. Luciferase signals were quantified with a software-assisted region-of-interest (ROI) analysis and foods were categorized into three main classes regarding

their toxin formation capability: high-risk, risk, and low-risk foods (Table 2, Figures S1–S3 in Supplementary Material available online at <http://dx.doi.org/10.1155/2014/465603>). Derived mean ROI values of each risk category and the corresponding determined threshold values are listed in Table S1. The bioassay revealed that 44% of the foods could be categorized as high-risk foods, while the remaining 20% and 36% were categorized as risk or low-risk foods, respectively (Table 2). Products classified as being insensitive were dairy based, displayed a low pH value (e.g., cream cheese and unsweetened quark), had a high fat content like chocolate and nut spread, and/or were characterized by low water availability or high osmolarity (e.g., dried fruits and 10% herbal salt solution). Earlier studies showed that growth of *B. cereus* was suppressed in foods with pH values below 5.0 [34–36], which is in line with our low-risk classification of matrices that had pH values around 4.3 to 4.8, such as the whey drinks. The combination of neutral pH values and medium a_w -values with high amounts of fat and cocoa was found to be indicative of the group of products being at medium risk of toxin synthesis (Table 2 and Figure S2). Likewise, proteinaceous foodstuff containing high fat amounts, such as minced beef or milk powder-based processed cheeses, fell in the same category. This is in agreement with a previous study [37] showing that cereulide was produced in small quantities in artificially contaminated meat products. The same study also supports our results concerning the pasteurized milk: usually, only low to medium cereulide levels are produced under stationary conditions at room temperature [30, 37]. Additionally, dairy products dulcified with glucose or fructose (quark desserts, cream-filled soft biscuits) fell in the intermediate class in terms of the risk for cereulide production. It was shown previously that glucose had a stimulating effect on cereulide synthesis [4]. The group of high-risk products comprised farinaceous foods, as well as powdered products that were reconstituted with water or milk (Table 2, Figure S3). Dairy- and cereal-based infant food formulas, which were additionally enriched with vitamins or trace elements, promoted exceptional high *ces* promoter activities. The latter indicates that a combination of readily available saccharides, vitamins, and macronutrients in a pH neutral environment may stimulate toxin formation. Indeed, cereulide was detected in high levels in farinaceous matrices or systems containing high amounts of K^+ ions and vitamins [10, 38].

A summary of food characteristics commonly observed in the three categories is provided in Figure 3. This generalized scheme allows a basic preevaluation of foods and their ingredients concerning their capability to support cereulide formation and should facilitate hazard identification in terms of HACCP concepts.

4. Conclusion

Overall, our results indicate that emetic *B. cereus* strains occur more frequently and in a much broader diversity of foods than noticed so far. In addition, the *lux*-based real-time monitoring assay turned out to be a valuable tool for

assessing the actual risk of cereulide toxin production in different types of food, allowing us to set up a general scheme for the categorizing of foods with respect to their cereulide production risk. Our survey of presumptive emetic *B. cereus* foodborne outbreaks also showed that the risk of an emetic syndrome caused by the *B. cereus* cereulide toxin is not restricted to high-carb foods, such as pasta and rice. Much more attention must be paid to other foods, especially the ones supporting cereulide production, as shown by the *lux* reporter assay.

Conflict of Interests

The authors have declared no conflict of interests.

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Research Article

Genomic and Proteomic Characterization of Bacteriocin-Producing *Leuconostoc mesenteroides* Strains Isolated from Raw Camel Milk in Two Southwest Algerian Arid Zones

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Information on the microbiology of camel milk is very limited. In this work, the genetic characterization and proteomic identification of 13 putative producing bacteriocin *Leuconostoc* strains exhibiting antilisterial activity and isolated from camel milk were performed. DNA sequencing of the 13 selected strains revealed high homology among the 16S rRNA genes for all strains. In addition, 99% homology with *Leuconostoc mesenteroides* was observed when these sequences were analysed by the BLAST tool against other sequences from reference strains deposited in the Genbank. Furthermore, the isolates were characterized by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) which allowed for the identification of 2 mass peaks 6242 m/z and 5118 m/z that resulted to be specific to the species *L. mesenteroides*. Remarkably, the phyloproteomic tree provided more intraspecific information of *L. mesenteroides* than phylogenetic analysis. Accordingly, phyloproteomic analysis grouped *L. mesenteroides* strains into different subbranches, while all *L. mesenteroides* isolates were grouped in the same branch according to phylogenetic analysis. This study represents, to our knowledge, the first report on the use of MALDI-TOF MS on the identification of LAB isolated from camel milk.

1. Introduction

Increasing consumer demand for natural, healthy, and convenient foods has resulted in a new generation of minimally processed foods that focus on biopreservation, refrigeration, and packaging as hurdle strategies to extend the shelf-life of these products. The use of natural antimicrobial metabolites from lactic acid bacteria (LAB) has been determined to be one of the most promising strategies in minimal processing. LAB are food-grade organisms that may be used as an alternative to chemical preservatives in biopreservation strategies due to their ability to produce several antimicrobial compounds, including organic acids, hydrogen peroxide, and

bacteriocins [1]. *Leuconostoc* spp. and other LAB strains isolated from meat or dairy products produce bacteriocins that are active against the major food pathogen *Listeria monocytogenes* [2–6]. Although this activity was first observed in the 1950s, extensive studies on bacteriocins produced by *Leuconostoc* spp. have only been conducted in the last 25 years. The importance of *Leuconostoc* strains in the dairy industry is widely recognized; however, knowledge of their physiology and genetics is less developed than that of *Lactococcus* [7].

Traditional dairy products such as LAB represent a reservoir of phenotypic and genetic microbial diversity, which may have biotechnological applications [8–10]. To date, raw camel's milk has been underinvestigated as a potential source

of food-grade LAB and has not generated a large industrial interest. One of the main reasons for the underinvestigation of raw camel milk is that the world production of camel milk for human consumption was recently estimated to only be 1.3 million tons/year [11]. Algeria produces only 8.100 tons/year of camel milk, but other countries such as Saudi Arabia (90.000 tons/year) and Sudan (82.250 tons/year) are strong producers. The majority of scientific studies on camels have been mainly focused on their anatomic characteristics and physiological adaptation to adverse climates. Consequently, information regarding camel milk is very limited. Previous studies on the molecular characterization of LAB isolated from fermented camel milk have been reported in the Xinjiang region of China [12], on the isolation of *Lactococcus lactis* from Algerian camel milk [13] and on the isolation of *L. mesenteroides* from fermented camel milk, "Raib" [14]. However, *L. mesenteroides* strains isolated from raw Algerian camel milk have not been characterized.

In the present study, raw camel milk was chosen because of its beneficial effects on human health [15], such as its antibacterial activity [16], antiviral activity [17] (Redwan and Tabl 2007), anti-inflammatory activity [18], anticancer activity [19], and antiallergic activity [20]. Additionally, camel milk is known for its extended shelf-life, which allows for storage and safe consumption after several days in the absence of refrigeration [21].

Leuconostoc and other LAB traditionally have been characterized phenotypically. However, new molecular techniques have been proposed for *leuconostocs* and other LAB to avoid the limitations of phenotypic characterization to achieve reliable and consistent identification. Therefore, 16S rRNA-based amplification and sequencing methods have been reported for the characterization of *leuconostocs* by Lee et al. [22], Schönhuber et al. [23], Pérez et al. [24], Randazzo et al. [25], Dal Bello et al. [26], Ennahar et al. [27], Kim et al. [28], and Reeson et al. [29]. More recently, proteomic tools such as matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) have also been proposed for bacterial identification purposes. These proteomic tools offer high throughput (95%–97.4% correct identifications) [30, 31] and produce unprecedented levels of discrimination among bacterial species and strains [32–35]. Therefore, the objective of this study was to isolate and identify *L. mesenteroides* strains exhibiting antibacterial activity from Algerian raw camel milk and use MALDI-TOF MS to determine protein biomarkers useful for the specific identification and classification of *L. mesenteroides*.

2. Material and Methods

2.1. Raw Camel Milk Sampling. The 13 *Leuconostoc* strains considered in this work were isolated from four different camel milk samples, which were collected at two different sampling times (2009 and 2011) from two different Algerian arid zones situated in the southwest of Algeria. The first zone, called Nâama, is located at 432 km away from the capital Algiers; the second zone, Abadla, close to the city of Béchar, is situated at 1150 km away from the capital. The first two

samples were collected from two camels (*Camelus dromados*) in Nâama which were in the range of 10–15 years old and coloured in grey and black, respectively. Both camels had the same lactation period, which was in March 2011. The diet of these camels was based on natural Saharan plants, called drinn (*Aristida pungens*). Samples from Béchar were collected at Abadla in 2009 and 2011 from brown camels aged less than 10 years that have a daily production of 6 to 9 milk liters. In all cases, sampling was performed under aseptic conditions by washing the teats with warm water containing 2% bleach and collecting milk in sterile glass bottles after hand washing with diluted alcohol. Samples were then transported by airplane to the laboratory in a cool box and stored at $4 \pm 1^\circ\text{C}$ until analysis. The samples were analyzed within 12 to 30 hrs after collection.

2.2. Bacterial Strains and Culture Conditions. The bacteriocin-producing *leuconostocs* considered in this work were isolated from raw camel milk as described above. All strains were stored at -80°C in reconstituted skimmed milk containing 30% (w/v) glycerol. All strains were cultured in MRS broth (Liofilchem, Teramo, Italy) at 30°C for 24 h and were then seeded onto MRS agar (Liofilchem) to obtain single colonies. Ten wild-type and reference *leuconostoc* strains used in this study are shown in Table 1. Thus, five reference strains were considered: three from the Spanish Type Culture Collection and two from the Ghent University Type Culture Collection (Table 1).

2.3. Phenotypic Characterization of Isolates. Fifteen strains were selected and subjected to the following physiological tests on the basis of the following phenotypic and morphological criteria: CO_2 production, growth at different temperatures (4°C , 15°C , 30°C , 37°C , and 45°C), growth at different pH (4.8 and 6.8), and growth at different NaCl concentrations (3% and 6.5%). Additionally, all strains were subjected to the following biochemical tests in order to differentiate between *leuconostocs* and *lactobacilli*: dextran production on MSE medium [36], arginine hydrolysis on M16BCP medium (Oxoid Ltd., London, UK), and citric acid degradation on Kempler and McKay solid medium. Carbohydrate fermentation was performed on MRS supplemented with bromocresol purple as a pH indicator by using the following sugars to differentiate between the following subspecies of *leuconostocs*: arabinose, maltose, rhamnose, esculine, mannitol, sorbitol, galactose, lactose, fructose, glucose, sucrose, and xylose. All strains considered in this study were phenotypically identified as belonging to the *Leuconostoc* genus based on the following criteria: ovoid shape, Gram-positive, catalase negative, vancomycin-resistant, production of gas from glucose, no arginine hydrolysis, and by their fermentation profiles.

2.4. Inhibition Assays of Indicator Microorganisms. Preliminarily, all strains were tested for their ability to produce antimicrobial substances by the direct method described by Fleming et al. [37]. Inhibitory activity was investigated on the following indicator bacteria: *Lactobacillus plantarum*,

TABLE 1: Reference strains considered in the phylogenetic and proteomic studies.

Species	Source	Origin	Code
<i>Leuconostoc pseudomesenteroides</i>	CECT 4027	Juice	L.PSEUD_CECT_4027
<i>Leuconostoc mesenteroides</i>	CECT 219	Fermented olives	L.MESEN_CECT_219
<i>Leuconostoc carnosum</i>	CECT 4024	Beef meat	L.CARNO_CECT_4024
<i>Leuconostoc mesenteroides</i>	LMG 6908	ND	L.MESEN_LMG_6908
<i>Leuconostoc pseudomesenteroides</i>	LMG 11482	ND	L.PSEUD_LMG_11482
<i>Lactococcus lactis</i>	LHICA	Cow milk	Lc.LACTI.LHICA_30
<i>Lactococcus lactis</i>	LHICA	Cow milk	Lc.LACTI.LHICA_31
<i>Lactococcus lactis</i>	LHICA	Cow milk	Lc.LACTI.LHICA_33
<i>Lactococcus lactis</i>	LHICA	Cow milk	Lc.LACTI.LHICA_63
<i>Leuconostoc 23.3</i>	LHICA	ND	L.MESEN.LHICA.Z_23.3

CECT: Spanish Type Culture Collection; LMG: Ghent University Type Culture Collection; LHICA: University of Santiago LHICA Bacterial Collection; and ND: not determined.

Lactococcus sp. (LMA, Oran, Algeria), *Escherichia coli*: 25922, *Staphylococcus aureus*: 43300 (Centre Hospitalier Universitaire, C.H.U Oran, Algeria), *Listeria innocua* (ATCC 33090), and *Listeria ivanovii* (ATCC 19119). Aliquots of 18 h cultures of each *Leuconostoc* strain were spotted on MRS agar using multipoint inoculators and were incubated at 30°C for 24 h [38]. Following incubation, a semisolid Mueller Hinton (Oxoid) medium containing 100 µL of 10⁷ CFU mL⁻¹ of indicator culture was poured as an overlay. All plates were then incubated at 37°C for 24 h and examined for the formation of inhibition zones. Inhibition was considered positive when the width of the clear inhibition halos was ≥ 0.5 cm.

2.5. Genetic Identification of *Leuconostoc* Strains, Phylogenetic Analysis, and Clustering. Total genomic DNA was extracted and purified using the DNeasy Tissue Mini Kit (Qiagen, Valencia, CA) [39]. Briefly, this method utilized the purification of DNA using microcolumns and its final recovery using a commercially prepared elution buffer. A fragment of the 16S rRNA gene was amplified by PCR using the universal primer pair p8FPL (forward: 5'-AGTTTGATCCTGGCTCAG-3') and p806R (reverse: 5'-GGACTACCAGGTATCTAAT-3') [40]. All PCR assays were conducted on a "My Cycler" Thermal Cycler (BioRad Laboratories, Hercules, USA). The assays comprised 100 ng of template DNA, 25 µL of a master mix (BioMix, Bioline, London, UK) (this included the reaction buffer, dNTPs, and magnesium chloride), Taq DNA polymerase, 25 pmol of each oligonucleotide primer, and double-distilled water to achieve a final volume of 50 µL. Amplification conditions were as follows: denaturing at 94°C for 7 min, 35 cycles of denaturation (94°C for 60 sec), annealing (55°C for 60 sec), extension (72°C for 60 sec), and a final extension at 72°C for 15 min. The PCR was performed as described by Böhme et al. [41].

Prior to sequencing, PCR products were purified with the "EXOSAP-IT" Kit (GE Healthcare, Uppsala, Sweden). Direct sequencing was performed with the "Big Dye Terminator v 3.1" Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The same primers used for PCR were also used for sequencing both strands of the PCR products.

The sequencing reactions were analysed in an automatic sequencing system (ABI 3730XL DNA-Analyzer, Applied Biosystems) with the POP-7 system. All 16 rRNA gene sequences were analysed with Chromas software (Griffith University, Queensland, Australia) and aligned using Clustal X software [42]. Following alignment, these sequences were identified by searching for sequence homology among published reference sequences using the web BLAST tool (National Center for Biotechnology Information (NCBI), <http://blast.ncbi.nlm.nih.gov/>) [43]. Homologies higher than 99% with respect to a strain type were considered good identifications.

Phylogenetic and molecular evolutionary analyses were conducted with MEGA 5.0 software [44]. Phylogenetic clustering and construction of a phylogenetic-based tree were performed using the neighbour-joining method [42] by using the "Bootstrap method" as a test of phylogeny and the "Kimura 2-parameter model" to compute the evolutionary distances [45, 46]. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed [47]. Meanwhile, estimates of evolutionary divergence and diversity values for 16S rRNA gene sequences were conducted with the MEGA 5.0 software using the "Maximum composite likelihood model" [46-48].

2.6. MALDI-TOF MS and Phyloproteomic Analysis of *Leuconostoc* Isolates. The 13 *Leuconostoc* strains whose 16S rRNA had been sequenced were grown on MRS agar plates for 24 h. Then, a 1 µL loop of each bacterial culture was harvested and placed in 100 µL of a solution consisting of 50% acetonitrile (ACN) (Merck, Darmstadt, Germany) and 1% aqueous trifluoroacetic acid (TFA) (Acros Organics, Morris Plains, NJ). The bacterial pellet was vortexed at least two times until the pellet was completely resuspended. Complete homogenization of the mixture was required to obtain good spectral profiles for *leuconostocs*. After centrifugation at 8000 rpm for 10 min, the supernatants were transferred to new tubes and stored at -20°C. A 1 µL aliquot of each sample solution was mixed with 10 µL of a matrix solution consisting of 10 mg α-cyano-4-hydroxycinnamic acid (α-CHCA) in 1 mL of 50% ACN and 2.5% aqueous TFA. From this final solution of sample and

TABLE 2: Fermentation profiling of *Leuconostoc* strains isolated from camel milk.

Strains	Ara	Mal	Rha	Esc	Man	Sorb	Gal	Lac	Fru	Glu	Sac	Xyl
Z.1-09	+	-	+/-	+/-	-	-	+	+	+	+	+	+
Z.2-09	+	+	-	+	+	-	+	+	+	+	+	+
Z.3-09	+	+	-	-	+	-	+	+	+	+	+	+
Z.4-09	+	+	-	+	+	+/-	+	+	+	+	+	+
Z.5-09	+	+	-	+	+	+	+	+	+	+	+	+
Z.6-09	+	+/-	-	+/-	-	+/-	+	+	+	+	+	+
zB7	-	+	-	-	-	-	+	+	+	+	+	+
Z.R1	+	+	NI	-	-	-	+	+	+	+	+	+
Z.R2	+	+/-	NI	-	-	-	+/-	+	+	+/-	+/-	+
Z.R3	+	+/-	NI	+/-	+/-	-	+/-	+	+	+	+/-	+/-
Z.R4	+	+/-	NI	-	-	-	+	+	+	+/-	-	+
Z.R5	-	+	NI	-	-	-	+	+	+	+	+	+
Z.R6	+	+/-	NI	-	+/-	-	+	+	+	+	+	+

NI: not identified.

TABLE 3: Diameters of the inhibition zones of *Leuconostoc* strains (Z1.09 to B7) isolated from camel milk on indicator strains.

Strains	Z.1-09	Z.2-09	Z.3-09	Z.4-09	Z.5-09	Z.6-09	R1	R2	R3	R4	R5	R6	B7
<i>Lactobacillus plantarum</i>	7	8	11	8	9	10	NI	NI	NI	NI	NI	NI	9
<i>Lactococcus</i> sp.	8	6	8	8	7	7	20	17	20	20	20	20	5
<i>Escherichia coli</i>	8	9	7	10	10	8	15	12	16	20	18	17	7
<i>Staphylococcus aureus</i>	8	8	7	9	11	8	18	20	25	27	17	20	8
<i>Listeria innocua</i>	8	6	9	9	8	10	10	11	10	10	8	10	9
<i>Listeria ivanovii</i>	8	9	7	9	11	10	10	7	6	11	-	-	8

matrix, a 1 μ L aliquot was manually deposited onto a stainless steel plate and allowed to dry at room temperature.

Mass spectra were obtained using a Voyager “DE STR MALDI-TOF” Mass Spectrometer (Applied Biosystems, Foster City, CA) operating in a linear mode and extracting positive ions with an accelerating voltage of 25,000 V and delay time of 350 ns. The grid voltage and guide wire were set to 95% and 0.05%, respectively. Each spectrum was the accumulated sum of at least 1000 laser shots, which were obtained from 10 different regions and manually selected from the same sample spot in a range of 1500–15000 Da. For every strain, two extractions were performed and both extracts were measured in duplicate totalling of four spectra for each bacterial strain. The mass spectra were externally calibrated using a mixture of 1 pmol/ μ L oxidized insulin B-chain and 1 pmol/ μ L bovine insulin (Sigma-Aldrich, St. Louis, MO) that were analysed with Data Explorer software (version 4.0) (Applied Biosystems) for baseline correction and noise filtering.

After obtaining four spectral profiles for each bacterial strain, mass spectra were analysed with Data Explorer software (version 4.0), baseline corrected, and noise filtered. Data lists containing m/z values were extracted from mass spectral data, including signals with relative intensities higher than 2%. The obtained peak mass lists were analysed and compared using peaks in the mass range of 2000–10000 Da because of the reproducibility of the spectral profile in that mass range.

Mass lists were further processed with the free web-based application SPECLUST, which is available at “<http://bioinfo.thep.lu.se/speclust.html>” [49]. The “peaks in

common” option in this web interface calculates the mass difference between four peaks taken from different peak lists and determines if two peaks are identical after taking into account measurement uncertainty (σ) and peak match score (s). The peak match score represents the probability that two peaks with measured masses m and m' have a mass difference equal or larger than $|m - m'|$ given that the mass difference is only due to measurement errors. Because each bacterial strain was cultured in duplicate and each culture was analysed in duplicate, this tool was used to examine the four spectra from each sample. The representative common peaks present in all four spectra were extracted by this web application with a peak match score greater than 0.7 (which corresponds to a measurement error of ± 5 Da) to obtain species-specific and genus-specific biomarkers. A peak was considered to be common to four spectra if the peak match score was larger than 0.7, which corresponded to a range in peak match score of 10 Da. According to these specifications, specific mass lists were generated for every bacterial strain (including 5–35 peak masses), which represented reproducible bacterial fingerprints.

Mass lists of all *leuconostocs* were clustered by using the “clustering” option that is also available in the web interface SPECLUST. The agglomerative clustering method created one cluster for every peak list and calculated distances between clusters. The two clusters with the smallest maximum pairwise distance (complete linkage) were then merged into a new cluster and the distances were calculated again by adding all individual similarity scores for every pair of two peak lists. This process was repeated until one single

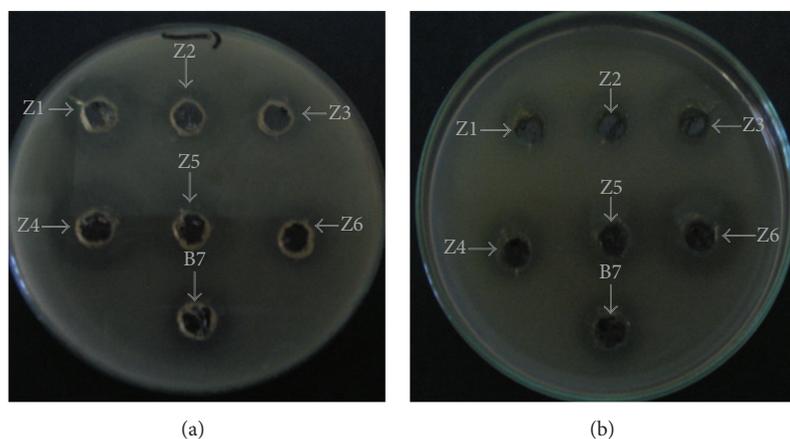


FIGURE 1: Antibacterial activity after eliminating lactic acid with buffered medium. (a) *Leuconostoc mesenteroides* effect on *Listeria innocua*. (b) *Leuconostoc mesenteroides* effect on *Listeria ivanovii*. Z1–Z6: *L. mesenteroides* isolated from camel milk sample 1 and B7: *L. mesenteroides* isolated from camel milk sample 2.

cluster remained. All individual similarity scores of each pair of the two peak lists were added up to calculate the distances between the two peak lists. The width in the peak match score was set to 10 Da. Resulting distances varied between “1” for completely different set of peak masses and “0” for perfect matches.

Finally, phyloproteomic clustering was confirmed through the analysis of mass lists using Statgraphics Plus software (version 5.1). The mass list table was transformed into a binary table, which was followed by clustering by using centroid and group average analytical methods and by using block population distance metric and cluster variable options.

3. Results

3.1. Phenotypic Characterization of *Leuconostoc* Isolates from Raw Camel Milk. Macroscopic observation of bacterial colonies led to the selection of 15 observably different 0.5–1.5-mm-wide white small colonies that had a lenticular shape on MRS agar supplemented with vancomycin. All of the colonies exhibited a glutinous transparent aspect on MSE agar. All 15 isolates were Gram-positive and catalase negative, exhibited ovoid shape, and were associated with short pairs and/or chains. Additionally, all isolates were citrate positive, were able to produce CO₂ from glucose, were able to produce dextran from sucrose, and were unable to hydrolyse arginine. Furthermore, all isolates were able to grow at 15°C, 30°C, and 37°C but were unable to grow at 4°C and 45°C. All isolates were resistant to 3% NaCl and to pH 6.8. None of the isolates were able to grow on 6.5% NaCl at pH 4.8. Fermentation profiling showed that the 13 strains that exhibited antilisterial activity were able to ferment glucose and lactose, but these strains exhibited some differences in their ability to ferment other sugars (Table 2).

3.2. Antimicrobial Activity of *Leuconostoc* Isolates. Thirteen of the 15 isolates exhibited inhibitory activity against other

LAB such as *Lactobacillus* spp. and *Lactococcus* spp. and against several pathogenic bacteria, such as *E. coli*: 25922, *S. aureus*: 43300, *L. innocua* (ATCC 33090), and *L. ivanovii* (ATCC 19119). The inhibition zones were measured and their diameters are compiled in Table 3. The results of inhibition indicated that the inhibition intensity and range varied depending on the *leuconostoc* species assayed.

Furthermore, to investigate whether the cause of the inhibition was due to protein, buffered supernatants adjusted to pH 6.8 were treated with chymotrypsin, which lead to the disappearance of inhibition zones. This result indicated that inhibition was caused by a proteinaceous compound (Figure 1). However, inhibition remained after heating the bacterial supernatants to a temperature of 100°C (data not shown), which indicated that the causative inhibitory agent is heat resistant. These results agreed with previous results reported by Lachance [50] and Labioui et al. [51].

3.3. Phylogenetic Analysis of *Leuconostoc* Isolates. DNA sequencing of the 13 selected isolates revealed high homology among their 16S rRNA nucleotide sequences. In addition, sequence analysis by the BLAST tool against other sequences from reference strains deposited in the GenBank revealed a 99% homology with *L. mesenteroides*. A phylogenetic tree was constructed by considering other *Leuconostoc* and *Lactococcus* reference and collection strains. These results are presented in Figure 2. Thus, phylogenetic analysis indicated that all strains isolated from raw camel milk were grouped in a common branch with reference strains *L. mesenteroides* (CECT 219) and *L. mesenteroides* (LMG 6908). This confirmed the identity of such strains as *L. mesenteroides*. However, *Leuconostoc pseudomesenteroides* (LMG 11482) and *L. pseudomesenteroides* (CECT 4027) were grouped together but were not in the same cluster as the *L. mesenteroides* strains. Additionally, *Leuconostoc carnosum* (CECT 4024) clustered in another branch separate from the other two. *Lactococcus* strains clustered into two distinct subclusters corresponding to (i) *Lactococcus lactis* subsp. *cremoris*, which

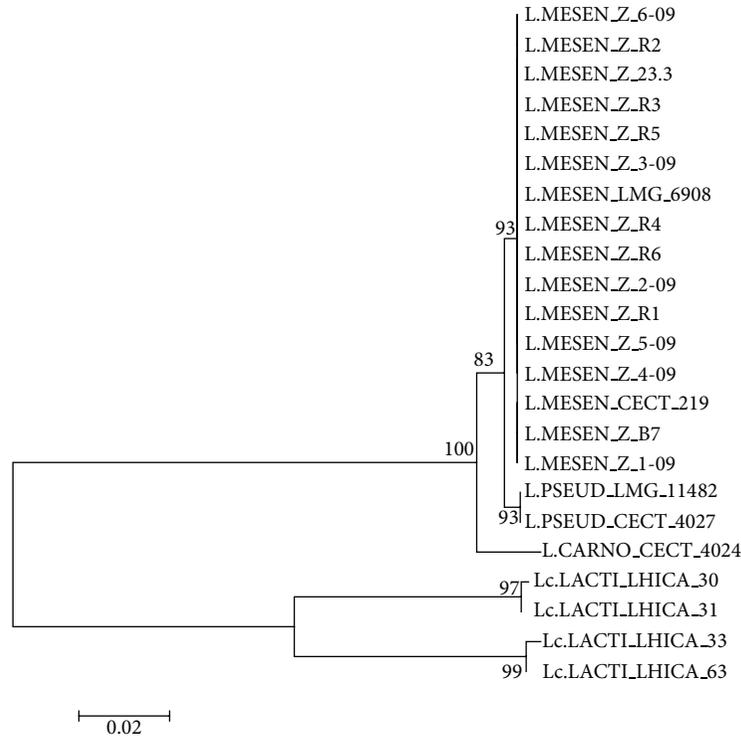


FIGURE 2: Phylogenetic tree of *Leuconostoc* spp. This tree was generated for *Leuconostoc* spp. isolated from camel milk and other reference strains based on 16S rRNA nucleotide sequences using the neighbour-joining method. Numbers above and below branches indicate bootstrap values from the neighbour-joining analysis.

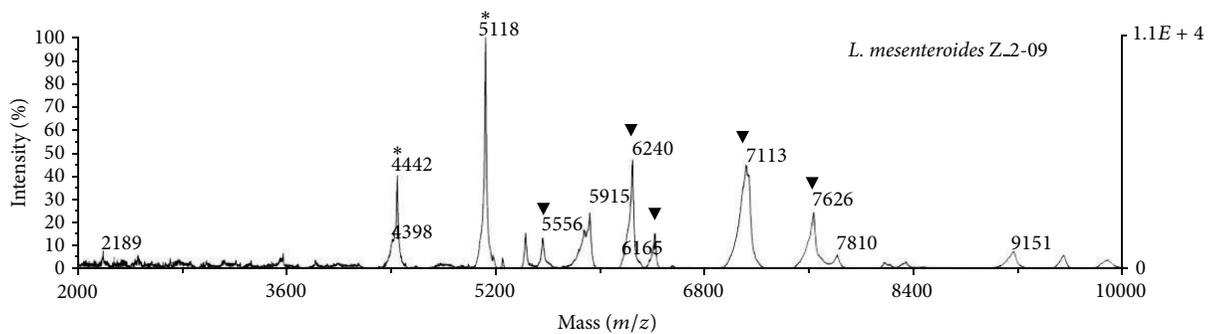


FIGURE 3: Characteristic mass peaks of *L. mesenteroides* strain Z.2-09 isolated from camel milk. Specific genus peaks marked as (*) are present in other *Leuconostoc* strains and peaks marked as (▼) are absent in the *Lactococcus* genus.

included the *L. lactis* (LHICA 63) and *L. lactis* (LHICA 33) strains, and (ii) *L. lactis* subsp. *lactis*, which included the *L. lactis* (LHICA 30) and *L. lactis* (LHICA 31) strains. These two subclusters were separated by a short distance due to their high genetic similarity as compared to *L. carnosum*, *L. mesenteroides*, and *L. pseudomesenteroides*.

3.4. MALDI-TOF MS Fingerprinting of *Leuconostoc* Isolates. Identification of *leuconostocs* was also performed by MALDI-TOF MS. Four spectra were obtained for each strain. The search for common peak masses in the spectra was performed using the SPECLUST application. Arithmetic means were

calculated for m/z values and the standard deviation was calculated to be ± 5 Da. The mass lists include 68 peak masses that were generated for 19 *Leuconostoc* strains with four *Lactococcus* strains classified as an outgroup. While 46 peaks were only observed in *Leuconostoc* strains, 20 peaks were specific to the *Lactococcus* genus and only two peaks were shared by both genera.

Remarkably, the spectral profile (fingerprinting) revealed different results for *Lactococcus* and *Leuconostoc* genera. The highest intensity peak in *Lactococcus* appeared at m/z 3865 Da, while the highest intensity peak in *Leuconostoc* appeared at m/z 5118 Da. Significant differences in the mass peak lists between these two genera were observed (Figure 3).

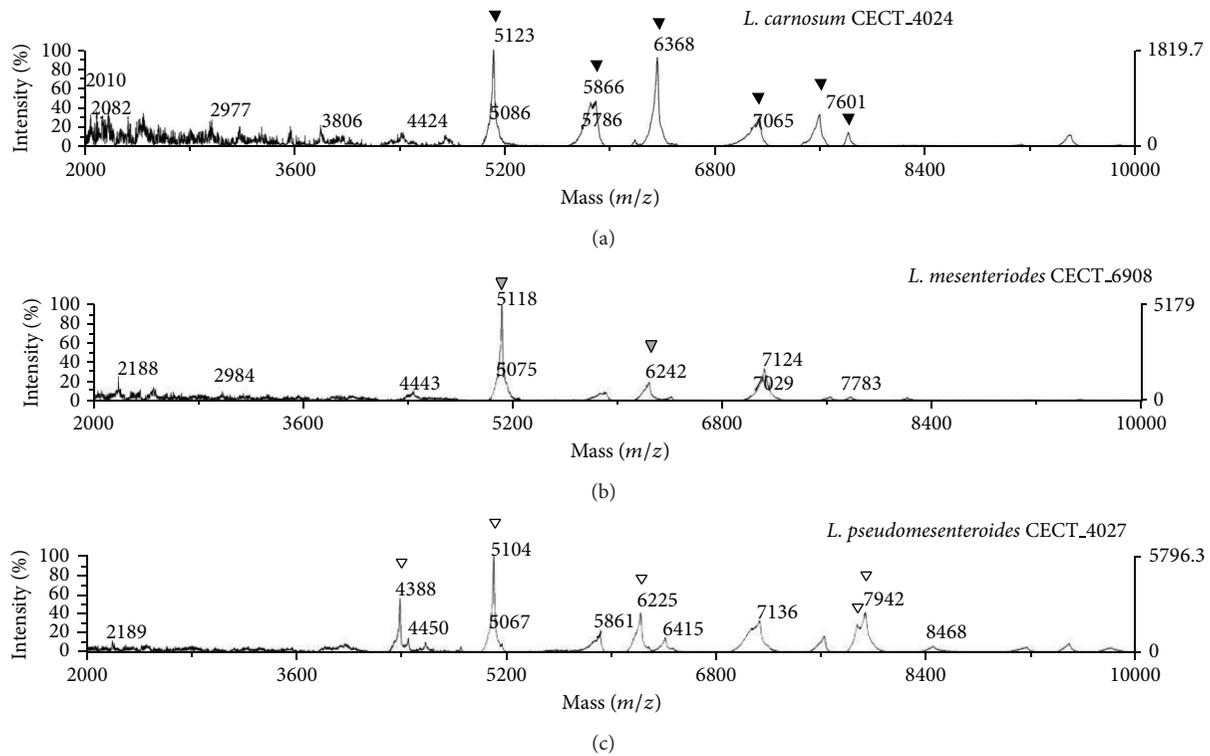


FIGURE 4: Spectra corresponding to the species *L. carnosum*, *L. mesenteroides*, and *L. pseudomesenteroides*. Species-specific peaks are marked in each case by (▽).

The spectral profiles for the different *Leuconostoc* spp. shared a great number of peaks, but there were some differences in the presence/absence of peaks (Figure 4). Thus, the peak at m/z 6242 Da was present in both *L. mesenteroides* and *L. pseudomesenteroides* but was shifted at 6368 Da in *L. carnosum*. Of the 46 peaks present in the *Leuconostoc* genus, 10 were present in more than 50% of the samples analysed. It should be noted that the peak at m/z 4442 Da was present in all *Leuconostoc* spp. with the exception of strain RL, which is probably due to slight differences in the protein amino acid sequence [52]. Therefore, the peaks at m/z 4442 Da and m/z 5118 Da are specific for the *Leuconostoc* genus (Table 4). A phyloproteomic tree was constructed from the peak mass list (Figure 3) by using the SPECLUST program to differentiate between the *Leuconostoc* spp. isolated from raw camel milk. Thus, two main clusters were observed in the dendrogram: one cluster corresponded to the *Lactococcus* genus, which was considered an outgroup, while the other cluster included all three *L. carnosum*, *L. pseudomesenteroides*, and *L. mesenteroides* species. Remarkably, the phyloproteomic tree provided more intraspecific information for *L. mesenteroides* than 16S rRNA-based phylogenetic analysis. The phyloproteomic analysis allowed the *L. mesenteroides* strains to be grouped into different subbranches, while all *L. mesenteroides* isolates were grouped in the same branch according to phylogenetic analysis.

TABLE 4: List of species-specific peak masses of *L. carnosum*, *L. pseudomesenteroides*, and *L. mesenteroides*.

Microbial species		
<i>L. carnosum</i>	<i>L. pseudomesenteroides</i>	<i>L. mesenteroides</i>
4424	4388	6242
5123	5104	5118
5866	6225	
6368	7942	
7065		
7601		

4. Discussion

Camel milk is an important food in arid and semiarid regions where it covers most qualitative and quantitative nutritional needs. While many studies have investigated the microbiology of cow, sheep, and goat's milk, only a few studies have focused on the microbiology of camel milk. Other authors have reported the effectiveness of protective proteins from camel milk against bacteria, such as *L. lactis* subsp. *cremoris*, *E. coli*, *S. aureus*, *Salmonella typhimurium*, and *rotavirus* [16]. The inhibition of pathogenic bacteria by protective proteins such as lysozyme, lactoperoxidase, or lactoferrin naturally present in camel milk has also been previously described by Barbour et al. [53].

Remarkably, only a few studies have addressed the genetic identification of LAB isolated from camel milk and these

studies analysed other regions of the world [54–56]. Therefore, to the best of our knowledge, no genetic information regarding *Leuconostoc* spp. isolated from raw camel milk in northern Africa has been previously reported. Additionally, the use of MALDI-TOF MS for the characterization of LAB isolated from camel milk has never been performed before and only one study regarding the proteomic identification of *Leuconostoc* from other food sources has been performed by de Bruyne et al. [57]. The present study focused on the characterization and proteomic identification of *Leuconostoc* spp. from Algerian raw camel milk. *Leuconostoc* spp. act as starter cultures and also exert beneficial effects on the microbiological stability and production of aroma compounds in various food products. More importantly, *Leuconostoc* spp. play a crucial role in food biopreservation through the production of bacteriocins with different inhibition spectra (they are especially effective as antilisterial agents) [58].

The *L. mesenteroides* isolated in this work exhibited significant inhibition against indicator strains (Table 3). This inhibition was not caused by the production of organic acids, hydrogen peroxide, or lysogenic phages as the molecules responsible for inhibition were sensitive to protease treatment. Phenotypic, genotypic, and proteomic analysis revealed that the 13 *L. mesenteroides* isolates from raw camel milk were identical. Additionally, according to 16S rRNA gene sequencing, the 13 strains exhibited high similarity among themselves and with respect to other sequences from reference strains deposited in the GenBank. Moreover, phylogenetic analysis revealed that all 13 isolates clustered in the same branch, which confirms their clonal homogeneity.

Finally, this study represents the first report on the application of MALDI TOF MS analysis for the faster and more reliable identification of *L. mesenteroides* strains isolated from Algerian raw camel milk based on their low-molecular-weight protein profile. The spectra were generated in quadruplicate to ensure the reproducibility of these results. The small differences in the spectra of individual strains may be caused by bacterial response to stress and environmental changes, including storage and handling. Small spectral differences are observed in the majority of cases and can cause slight differences in the intensity of some peaks [59]; however, relevant peaks are rarely affected. This was also observed in our study (data not shown).

The mass spectrometry profiles obtained for each *Leuconostoc* sp. allowed for the generation of species-specific peak mass lists (Table 4) for *L. mesenteroides*, *L. pseudomesenteroides*, and *L. carnosum* (Table 1). Remarkably, these results allowed for the identification of peak masses specific to *L. mesenteroides* that could serve as biomarker peaks in future analyses. Moreover, the phyloproteomic tree proposed in this study for *Leuconostoc* spp. isolated from raw camel milk provided more intraspecific information than a 16S rRNA-based phylogenetic analysis of *L. mesenteroides*. Therefore, phyloproteomic analysis allowed for the grouping of *L. mesenteroides* strains into different subgroups (Figure 5), while the phylogenetic proximity of these strains did not allow for such differentiation [57].

In summary, this study has provided the first genetic characterization of bioactive *Leuconostoc* spp. isolated from

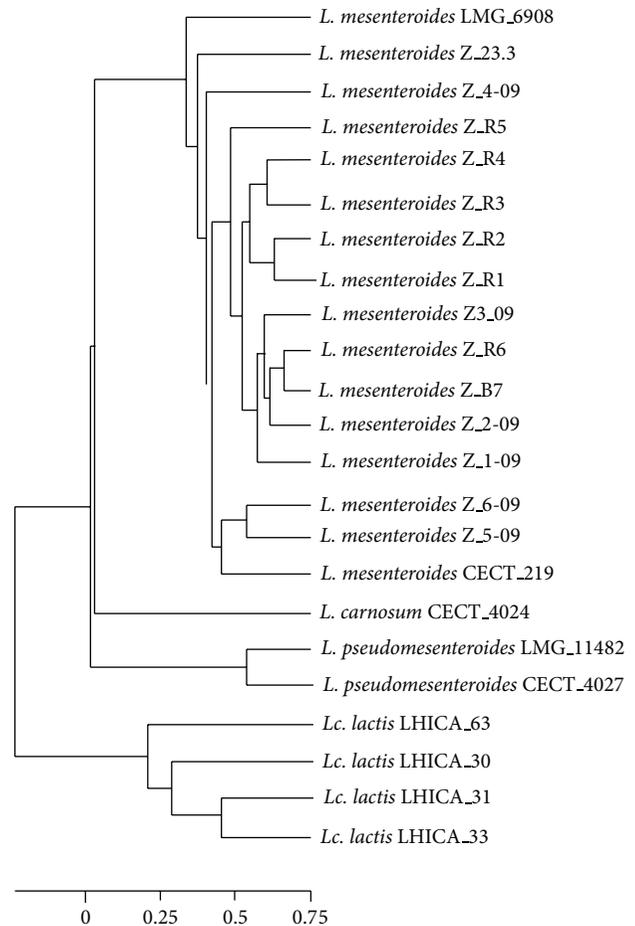


FIGURE 5: Phyloproteomic tree of *Leuconostoc* spp. based on the protein profile obtained by MALDI-TOF MS.

Algerian raw camel milk. Additionally, the application of MALDI TOF peptide mass fingerprinting was successfully applied to this bacterial group and proved to be a simple, quick, and inexpensive complementary method for bacterial identification at the species level.

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

***Staphylococcus aureus* and Staphylococcal Food-Borne Disease: An Ongoing Challenge in Public Health**

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Staphylococcal food-borne disease (SFD) is one of the most common food-borne diseases worldwide resulting from the contamination of food by preformed *S. aureus* enterotoxins. It is one of the most common causes of reported food-borne diseases in the United States. Although several Staphylococcal enterotoxins (SEs) have been identified, SEA, a highly heat-stable SE, is the most common cause of SFD worldwide. Outbreak investigations have found that improper food handling practices in the retail industry account for the majority of SFD outbreaks. However, several studies have documented prevalence of *S. aureus* in many food products including raw retail meat indicating that consumers are at potential risk of *S. aureus* colonization and subsequent infection. Presence of pathogens in food products imposes potential hazard for consumers and causes grave economic loss and loss in human productivity via food-borne disease. Symptoms of SFD include nausea, vomiting, and abdominal cramps with or without diarrhea. Preventive measures include safe food handling and processing practice, maintaining cold chain, adequate cleaning and disinfection of equipment, prevention of cross-contamination in home and kitchen, and prevention of contamination from farm to fork. This paper provides a brief overview of SFD, contributing factors, risk that it imposes to the consumers, current research gaps, and preventive measures.

1. Introduction

Food-borne diseases are a major public health concern worldwide [1, 2]. WHO defines food-borne disease (FBD) as “disease of infectious or toxic nature caused by, or thought to be caused by, the consumption of food or water” [2]. Annually, an estimated 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths are caused by food-borne diseases in the United States [3]. Among these cases, 31 known pathogens cause 9.4 million illnesses, 56,000 hospitalizations, and 1300 deaths [4]. Using data from 2000–2008, researchers estimated that pathogens that were implicated in most FBD were norovirus (5.5 million, 58%), nontyphoidal *Salmonella* spp. (1.0 million, 11%), *Clostridium perfringens* (1.0 million, 10%), and *Campylobacter* spp. (0.8 million, 9%). Among many food-borne pathogens, nontyphoidal *Salmonella* spp. and *Campylobacter* spp. are the leading causes of FBD in the United States, England, and Australia [4].

S. aureus is a significant cause of FBD, causing an estimated 241,000 illnesses per year in the United States

[4]. However, the true incidence of *Staphylococcus aureus* food-borne disease (SFD) could be a lot higher as sporadic food-borne disease caused by *S. aureus* is not reportable in the United States [5]. Some other contributing factors for the low incidence of SFD include misdiagnosis, improper sample collection and laboratory examination [6], lack of seeking medical attention by the affected persons complicating the laboratory confirmation [5, 7], and lack of routine surveillance of clinical stool specimens for *S. aureus* or its enterotoxins [5, 8, 9]. Unavailability of implicated foods for confirmation of laboratory testing at the time of outbreak investigation further complicates the matter [5]. It is essential to note that FBD that is confirmed by laboratory testing and reported to public health agencies accounts for only a small fraction of illnesses [4]. FBD impose a great economic burden, accounting for \$50–\$80 billion annually in “health care costs, lost productivity, and diminished quality of life” in the United States [10, 11]. It is estimated that each case of SFD costs \$695, representing a total cost of \$167,597,860 annually

in the United States [10]. The Institute of Medicine recognized FBD as a high priority [12]. “The potential for foods to be involved in the emergence or reemergence of microbial threats to health is high, in large part because there are many points at which food safety can be compromised.” Although FBD has decreased in recent years, it is still higher than *Healthy People 2020* goals [10]. The presence of food-borne pathogens in ready-to-eat foods, meat, and meat products puts consumers at high risk and imposes grave economic losses to producers due to recalls of implicated food products [13, 14].

2. *Staphylococcus aureus*

S. aureus is a commensal and opportunistic pathogen that can cause wide spectrum of infections, from superficial skin infections to severe, and potentially fatal, invasive disease [15]. This ubiquitous bacterium is an important pathogen due to combination of “toxin-mediated virulence, invasiveness, and antibiotic resistance.” This organism has emerged as a major pathogen for both nosocomial and community-acquired infections. *S. aureus* does not form spores but can cause contamination of food products during food preparation and processing. *S. aureus* can grow in a wide range of temperatures (7° to 48.5°C; optimum 30 to 37°C), pH (4.2 to 9.3; optimum 7 to 7.5), and sodium chloride concentration up to 15% NaCl. *S. aureus* is a desiccation tolerant organism with the ability to survive in potentially dry and stressful environments, such as the human nose and on skin and inanimate surfaces such as clothing and surfaces [16]. These characteristics favor growth of the organism in many food products [2]. *S. aureus* can remain viable on hands and environmental surfaces for extended durations after initial contact [17, 18].

3. Staphylococcal Food-Borne Disease

SFD is one of the most common FBD and is of major concern in public health programs worldwide [1, 2, 19]. It is one of the most common causes of reported FBD in the United States [1, 20–22]. The first documented event of SFD due to the consumption of contaminated cheese was investigated by Vaughan and Sternberg in Michigan, USA, in 1884 [19]. A typical FBD caused by *S. aureus* has a rapid onset following ingestion of contaminated food (usually 3–5 hours). This is due to the production of one or more toxins by the bacteria during growth at permissive temperatures [2]. However, the incubation period of SFD depends on amount of toxin ingested [22]. Very small dose of SEs can cause SFD. For example, one report indicated that approximately 0.5 ng/mL concentration of SEs contaminated with chocolate milk caused a large outbreak [22, 23].

The onset of SFD is abrupt. Symptoms include hypersalivation, nausea, vomiting, and abdominal cramping with or without diarrhea. If significant fluid is lost, physical examination may reveal signs of dehydration and hypotension [1, 6, 22, 24]. Abdominal cramps, nausea, and vomiting are the most common [2]. Although SFD is generally

self-limiting and resolves within 24–48 hours of onset, it can be severe, especially in infants, elderly, and immune-compromised patients [1, 6, 22]. Antibiotics are not used for therapy [7]. Approximately 10% of individuals inflicted with SFD will present to a hospital [22, 24]. Management of SFD is supportive. The attack rate of SFD can be up to 85% [22]. *S. aureus* may not be detected by culture in the events when food is contaminated and toxin is formed prior to cooking [22, 25]. A study involving 7126 cases indicated that case fatality rate of SFD is 0.03%; all deaths were in elderly patients [22]. Recovery is complete in approximately 20 hours [22, 24].

The conclusive diagnostic criteria of SFD are based upon the detection of staphylococcal enterotoxins in food [26], or recovery of at least 10^5 *S. aureus* g⁻¹ from food remnants [19]. *S. aureus* enterotoxin can be detected on the basis of three types of methods: bioassays, molecular biology, and/or immunological techniques [19, 27]. Polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), and RT-quantitative PCR can be carried out to evaluate the toxic potential of strain [19]. The enzyme immunoassay and enzyme-linked fluorescent assay are the most commonly used immunological methods based on the use of anti-enterotoxin polyclonal or monoclonal antibodies [19]. Several molecular typing methods are widely used for the genetic characterization of *S. aureus* such as multilocus sequence typing, *spa* typing, SCC*mec* typing, and Pulse-field gel electrophoresis (PFGE). These techniques provide means to trace epidemiologically related strains leading to the tracking back to the origin of contamination [28]. However, these methods have variation in their discriminating powers and can be increased by combining the methods [29]. Molecular-based methods provide information about the source of contamination (human or animal origin). The PFGE and *spa* typing can be used alone or in association to gather the information regarding the origin of *S. aureus* contamination [19].

Various types of foods serve as an optimum growth medium for *S. aureus*. Foods that have been frequently implicated in SFD are meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, especially cream-filled pastries and cakes, and sandwich fillings [2, 6, 30]. Foods implicated with SFD vary from country to country, particularly due to variation in consumption and food habits [2]. If food is prepared in a central location and widely distributed, SFD outbreaks can have grave consequences impacting thousands of people. For example, over 13,000 cases of SFD occurred in Japan in 2000 as a result of contamination of milk at a dairy-food-production plant [22, 31].

4. *Staphylococcal aureus* Enterotoxins

S. aureus produces wide arrays of toxins. Staphylococcal enterotoxins (SEs) are a family of nine major serological types of heat stable enterotoxins (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and SEJ) that belong to the large family of pyrogenic toxin superantigens [1, 6]. Pyrogenic toxins cause superantigenic activity such as immunosuppression and nonspecific T-cell proliferation [2]. It is hypothesized that

superantigenic activity of SEs helps facilitate transcytosis that allows the toxin to enter the bloodstream, thus enabling it to interact with antigen-presenting cells and T cells leading to superantigen activity [1, 6, 19]. The majority of effects of SEs in SFD is believed to be triggered by initiating a focal intestinal inflammatory response due to their superantigenic activity or by affecting intestinal mast cells causing their degranulation [1, 22, 32].

SEs are highly stable and highly heat-resistant and resistant to environmental conditions such as freezing and drying [2, 19]. They are also resistant to proteolytic enzymes such as pepsin or trypsin and low pH, enabling them to be fully functional in the gastrointestinal tract after ingestion [2, 6]. The heat stability characteristic of *S. aureus* imposes a significant threat in food industries [1]. The mechanisms of SEs causing food poisoning are not clearly known. However, it is believed that SEs directly affect intestinal epithelium and vagus nerve causing stimulation of the emetic center [2, 19]. All staphylococcal enterotoxins cause emesis [22, 32]. An estimated 0.1 µg of SEs can cause staphylococcal food poisoning in humans [2].

SEs produced by some strains of *S. aureus* are the causative agents of SFD, and SEA is the most common toxin implicated in such events. SEA is highly resistant to proteolytic enzymes. SEA was recovered from 77.8% of all SFD outbreaks in the United States followed by SED (37.5%) and SEB (10%) [1, 6]. SEA is the most commonly found enterotoxin among SFD outbreaks in Japan, France, and UK [6]. However, SEC and SEE are also implicated with SFD. The outbreak of gastrointestinal illness via contaminated coleslaw in the United States was caused by SEC produced by methicillin-resistant *S. aureus* (MRSA) from an asymptomatic food handler [33]. SEC was linked to the SFD outbreak in 1980 in Canada [34]. SEC was also involved in the SFD outbreak during 2001–2003 in Taiwan [35] and 2009 outbreak in Japan [36]. *S. aureus* is often implicated with caprine mastitis [37]. In sheep, goats, and cattle, SEC was the predominant toxin type detected in *S. aureus* isolated from mastitis milk [38]. Other studies have documented SEC producers as the most prevalent enterotoxin-producing *S. aureus* isolated from goat's milk [39] and goat's skin of udder, teats, and milk [40]. Six SFD outbreaks in France in 2009 were caused by SEE present in soft cheese made from unpasteurized milk [26]. Although rare, SEE has also been implicated in the SFD outbreaks in USA and UK [6]. Various new SEs (SEG to SE/U2) have been identified. However, only SEH-producing strains have been involved in SFD outbreaks [6].

S. aureus can survive in multiple host species. Molecular typing such as multilocus sequence typing (MLST) has helped to gain insights about population structure of *S. aureus*. Studies have identified over 2200 sequence types (STs) of *S. aureus* using the MLST techniques. The STs can be grouped into clonal complexes (CC). Several studies have indicated that majority of the livestock-associated STs belong to a small number of animal-associated clones. For example, CC97, ST151, CC130, and CC126 are commonly found on bovine infections. CC133 are common among small-ruminants such as sheep or goats. ST1, ST8, CC5, ST 121, and ST398 are

found in human host species [41]. ST5 is predominant among poultry isolates [42]. CC133 and ST522 are mostly implicated with mastitis in sheep and goats. One Danish study indicated that ST133 was the predominant lineage in sheep and goats [42].

5. Contributing Factors

In the United States, approximately 30% and 1.5% of the population are colonized with methicillin-susceptible *S. aureus* (MSSA) [43] and MRSA, respectively, [43–45] with the most important site for colonization being the anterior nares (nostrils) [46]. While colonization itself does not harm the host, it is a risk factor for developing subsequent symptomatic infections [43, 47]. These colonized healthy persons categorized as persistent carriage and intermittent carriage serve as *S. aureus* carriers and are able to transmit the bacterium to susceptible persons [46].

S. aureus is a common causative agent of bovine mastitis in dairy herds. A study conducted in Minnesota to estimate the herd prevalence of *S. aureus* from bulk tank milk found that herd prevalence of MSSA and MRSA was 84% and 4%, respectively [48]. Other studies estimated that the prevalence of *S. aureus* in bulk milk tank was 31% in Pennsylvania and 35% in cow milk samples in Louisiana [48]. Studies from Argentina [49], Brazil [50], Ireland [51], and Turkey [52] have documented the presence of staphylococcal enterotoxin genes and production of SEs by *S. aureus* of bovine origin. The udders with clinical and subclinical staphylococcal mastitis can contribute to the contamination of milk by *S. aureus* via direct excretion of the organisms in the milk [38] with large fluctuations in counts ranging from zero to 10⁸ CFU/mL [53]. For example, cattle mastitis was the sole source of contamination in 1999 *S. aureus* outbreak in Brazil that affected 328 individuals who consumed unpasteurized milk [54]. Similarly, 293 *S. aureus* isolates were recovered from 127 bulk tank milk samples of goats and sheep from Switzerland [38]. Recently, *S. aureus* isolates were recovered from mammary quarter milk of mastitic cows and from bulk tank milk produced on Hungarian dairy farms indicating that *S. aureus* from infected udders may contaminate bulk milk and, subsequently, raw milk products [53]. However, *S. aureus* contamination in milk can occur from the environment during handling and processing of raw milk as well [53].

Improper food handling practices in the retail food industry are thought to contribute to a high number of FBD outbreaks [55]. Studies have indicated that the majority of FBD outbreaks result from such practices [55, 56]. It was reported that the hands of food handlers were implicated in 42% of food-borne outbreaks that occurred between 1975 and 1998 in the United States [55, 57].

In a recent study [13] investigating the microbiological contamination in ready-to-eat food products processed at a large processing plant in Trinidad, West Indies, *S. aureus* was the most common pathogen detected. *S. aureus* was isolated from precooked food samples of franks, bologna, and bacon and postcooked bologna and bacon. The overall prevalence of *S. aureus* detected in air, food, and environmental samples

was 27.1% (46/170). It was determined that the counts of *S. aureus* increased after heat treatment, and only postcooking environmental surfaces that came into contact with ready-to-eat foods that were contaminated with *S. aureus* during slicing and packaging harbored *S. aureus*. *S. aureus* was also frequently found on food handler's gloves [13]. Pathogenic microbes can adhere to the surface of the gloves worn by retail food employees and can serve as a source of cross-contamination if not changed frequently [55]. The practice of wearing gloves without proper hand washing can contaminate both the interior and exterior of the gloves. Hand washing is often neglected when gloves are used, which may promote rapid microbial growth on the hands as gloves provide a warm, moist environment for bacterial growth on the hands [55, 57]. Hand-washing, an easy method of preventing many microbial contamination, is too often forgotten [55].

The finding of high bacterial counts in the air and on food contact surfaces in the postprocessing environment is suggestive of cross-contamination of postcooked products and is the most important risk factor affecting microbiological quality of food [13]. A study [58] found that processed foods that require more handling during preparation are more vulnerable to *S. aureus* contamination [13]. Another study [59] demonstrated that increased human handling contributed to contamination by *S. aureus* in a pork processing plant.

Analysis of the data of FBD outbreaks reported to the Food-borne Disease Outbreak Surveillance System during 1998 to 2008 [5] indicate that meat and poultry dishes were the most common foods (55% of *S. aureus* outbreaks) reported in *S. aureus* outbreaks in the United States. Foods implicated with *S. aureus* outbreaks were most often prepared in a restaurant or deli (44%). Errors in food processing and preparation (93%) were the most common contributing factor in FBD outbreaks. Forty-five percent and 16% of these errors occurred in restaurants and delis and homes or private residences, respectively. The study identified various errors in food processing and preparation that include (i) insufficient time and temperature during initial cooking (40%) hot holding (33%) and reheating process (57%); (ii) prolonged exposure of foods at room or outdoor temperature (58%); (iii) slow cooling of prepared food (44%); (iv) inadequate cold holding temperatures (22%); (v) and preparing foods for extended periods of time prior to serving [5]. Cross-contamination in the vicinity of food preparation and processing was another contributing factor in *S. aureus* food-borne outbreaks. Insufficient cleaning of processing equipment or utensils (67%) and storage in contaminated environments (39%) were the most common errors reported [5].

6. Farm, Food, and Beyond

In recent years, a new strain of *S. aureus*, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), has been recognized as a novel pathogen that has become a rapidly emerging cause of human infections [60, 61]. LA-MRSA was first detected in 2005 in swine farmers and

swine in France and in The Netherlands [62–64]. Researchers have isolated LA-MRSA from number of countries in Asia [65–67], Europe [68–74], and North America [75, 76]. Studies have found increased human colonization and infection of LA-MRSA belonging to the multilocus sequence type 398 (ST398) lineages in livestock-dense areas in Europe [77–80]. Investigators in The Netherlands have shown that ST398 now accounts for 20% of human MRSA cases [81] and this strain accounts for 42% of newly detected MRSA in that country, suggesting that animals may be an important reservoir for human MRSA infections [77]. Compared to the general population, Dutch pig farmers are 760 times more likely to be colonized with MRSA [82].

In several studies, MRSA has been found at high levels on US and European farms and in commercially-distributed meats, emerging as a potential concern for meat handlers and consumers [28, 64, 68, 75–77, 83–88]. Several species of meat-producing animals are frequently implicated including pigs [68, 75, 76], poultry [89–91], and cattle [73, 92]. The presence of MRSA on raw retail meat products is well documented, with prevalence ranging from less than 1 percent in Asia [93, 94] to 11.9% in The Netherlands [95], with intermediate prevalence found in other studies [87, 96, 97]. A recent study carried out in the United States found that 45% (45/100) of pork products and 63% (63/100) of beef products tested in Georgia were positive for *S. aureus*. The MRSA prevalence in this study was 3% and 4% in retail pork and beef, respectively [28]. Another US study testing retail meat in Louisiana isolated MRSA from 5% (6/120) of meat samples tested, while 39.2% (47/120) of samples were positive for any type of *S. aureus* [87]. Very high prevalence of *S. aureus* (64.8%, 256/395) was observed on retail pork products collected from Iowa, Minnesota, and New Jersey [85]. The prevalence of MRSA in this study was 6.6%. Other studies in US have found *S. aureus* in 16.4% (27/165) and MRSA in 1.2% (2/165) of meat samples [84], multidrug resistant (MDR) *S. aureus* in 52% (71/136) of meat and poultry samples [86], and any *S. aureus* in 22.5% (65/289) and MRSA in 2% (6/289) of meat and poultry samples [88]. These studies provide some insights regarding the role of commercially distributed meat as a potential vehicle for *S. aureus* transmission from the farm into the general human population.

The first report of an outbreak of gastrointestinal illness caused by a community-acquired methicillin resistant *S. aureus* in the United States affected 3 members of the same family. Contaminated coleslaw from an asymptomatic food handler was the source of MRSA [6, 33]. All 3 members of the family who ate foods (shredded pork barbeque and coleslaw) 30 minutes after purchasing at a convenient-market delicatessen developed gastrointestinal symptoms. The *S. aureus* isolates recovered from the stool samples of the three ill family members and coleslaw and nasal swab of food preparer were identical in PFGE analysis. The implicated strain produced Staphylococcal toxin C and was identified as MRSA [33].

This outbreak provides an evidence of MRSA-contaminated foods as the vehicle in the clusters of illness affecting low-risk persons within the community. The food handlers involved in this outbreak had visited a nursing

home. It is important to note that many *S. aureus* isolates obtained as a part of outbreak investigation may not be tested for antibiotic susceptibility, as antibiotics are not used in the treatment regimen. As such, it is plausible that food-borne outbreak caused by methicillin-resistant strains of *S. aureus* may go unnoticed. Previously food has been implicated as a source of MRSA transmission in one outbreak of blood and wound infections in hospitalized immunocompromised patients [33, 98].

7. Gaps in Research

Many outbreak investigations successfully traced food handlers as a source of contamination matching the strains of *S. aureus* in food products and handlers. However, these retrospectively carried-out studies have some limitations and cannot ascertain that the handler was not also colonized due to the exposure to *S. aureus* contaminated food.

Although numerous studies have focused on documenting risk imposed by *S. aureus* toxins in food industry and consumers' health, little is known about the potential role of intact bacteria transmitted through the raw meat products and self-inoculation into the nasal cavity of food industry workers and consumers. Additionally, while research has shown the potential for transmission of *S. aureus* within the home setting [99, 100], the relationship of colonization and transmission of this organism to the food products brought into the home has not been investigated.

Several European studies investigating MRSA in retail meat found ST398 as the most common MRSA type [95, 97, 101]. It has been suggested that meat might be a potential vehicle for the transmission of ST398 from the farm into the community, but additional research needs to be carried out to test this hypothesis.

Researchers have isolated other non-ST398 strains of *S. aureus* such as ST8, a strain which includes USA 300, the primary cause of community-associated MRSA infections, from US swine farms [102] and retail meat [28, 84–88]. However, it is not clear whether human handlers played any role during the postslaughter processing for the contamination of meat positive for ST8. It is suggested that since *S. aureus* is also present in intestinal tract [103], raw meat may contain MRSA due to the carcasses contaminated with intestinal content during slaughtering process [95]. Finding of human-associated strains of MRSA from raw chicken meat in Japan and Korea provides some support to this hypothesis [89, 93, 94].

Only few studies have been conducted specifically to investigate the implication of MRSA in SFD [19]. Although MRSA was frequently isolated from food production animals and raw retail meat, the relevance of its contamination is unknown. Further study is warranted to investigate the likelihood of gastrointestinal colonization and extraintestinal infection subsequent to the consumption of foods contaminated with MRSA [104]. Since *S. aureus* isolates obtained from SFD outbreaks may not be tested for antibiotic susceptibility, the true prevalence of MRSA involved in SFD is unknown [33]. Since other Staphylococcal species are also

able to produce SEs and are not routinely tested, further research is warranted [2].

8. Prevention

SFD is preventable [10]. Consumers need to be aware of potential food contamination in home and during cooking in kitchen. Cooking food thoroughly is important, but preventing contamination and cross-contamination and maintaining critical points are the most effective ways to prevent SFD. Since research findings and outbreak investigations have suggested that SFD is largely due to faulty food handling practices, knowledge and skills in food industry workers are warranted. Nevertheless, public health intervention should be designed to prevent *S. aureus* from pre- and postslaughter in meat processing facilities. Public awareness regarding safe meat handling would help to prevent cross-contamination [104] as well as potential colonization of handlers from contaminated food products. Other public health interventions such as personalized and tailored food safety education program targeting diverse sociodemographic people could be a cornerstone in preventing the SFD outbreak [10].

1985's staphylococcal food poisoning due to contaminated chocolate milk in Kentucky, USA, and 2000's extensive outbreak of staphylococcal food poisoning due to contaminated low-fat milk in Japan, are the classical examples of SFD that illustrate the stability and heat resistance of SEs as well as the importance of illumination of any contamination sources during the processing and refrigeration of food and food ingredients. In both cases, high temperature used in pasteurization killed the bacteria but had no effect on SEs [2, 31].

The permissive temperature for the growth and toxin production by *S. aureus* is between 6°C and 46°C. Thus, the ideal cooking and refrigerating temperature should be above 60°C and below 5°C, respectively. A study reviewing the performance of domestic refrigerators worldwide found that many refrigerators were running above the recommended temperature [105]. Another study conducted in Portugal found that more than 80% of participants cleaned their fridge only monthly [106]. While these studies indicate the need of consumer awareness in food safety, other preventive measures such as the practice of serving food rapidly when kept at room temperature, wearing gloves, masks, hairnets during food handling and processing, frequent hand washing, good personal hygiene of food handlers, and use of "sneeze-bars" at buffet tables could help prevent SFD [22, 58].

Maintaining the cold chain is essential for preventing the growth of *S. aureus* in food products [5]. Other preventive measures such as control of raw ingredients, proper handling and processing, adequate cleaning, and disinfection of equipment used in food processing and preparation should be deployed [19, 104]. Strict implementation and adherence to the microbiological guidelines such as Hazard Analysis and Critical Control Points (HACCP), Good Manufacturing Practice (GMPs), and Good Hygienic Practices (GHPs) developed by World Health Organization and United States Food and Drug Administration can help to prevent *S. aureus* contamination [13, 107].

9. Conclusion

SFD is one of the most common causes of FBD worldwide. Outbreak investigations have suggested that improper handling of cooked or processed food is the main source of contamination. Lack of maintaining cold chain allows *S. aureus* to form SEs. Although *S. aureus* can be eliminated by heat treatment and by competition with other flora in pasteurized and fermented foods, respectively, SEs produced by *S. aureus* are still capable of causing SFD because of their heat tolerance capacity. This fact should be considered in risk assessment and devising appropriate public health interventions. Prevention of *S. aureus* contamination from farm to fork is crucial. Rapid surveillance in the event of SFD outbreak and ongoing surveillance for the routine investigation of *S. aureus* and SEs implicated in food products along with improved diagnostic methods could help to combat the SFD in 21st century. Recent findings of high prevalence of *S. aureus* including MRSA in raw retail meat impose a potential hazard to consumers, both as classic SFD and as a potential source of colonization of food handlers. Further study is required to fill the research gap.

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

A Novel Electronic Nose as Adaptable Device to Judge Microbiological Quality and Safety in Foodstuff

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This paper presents different applications, in various foodstuffs, by a novel electronic nose (EN) based on a mixed metal oxide sensors array composed of thin films as well as nanowires. The electronic nose used for this work has been done, starting from the commercial model EOS835 produced by SACMI Scarl. The SENSOR Lab (CNR-INO, Brescia) has produced both typologies of sensors, classical MOX and the new technologies with nanowire. The aim of this work was to test and to illustrate the broad spectrum of potential uses of the EN technique in food quality control and microbial contamination diagnosis. The EN technique was coupled with classical microbiological and chemical techniques, like gas chromatography with mass spectroscopy (GC-MS) with SPME technique. Three different scenarios are presented: (a) detection of indigenous mould in green coffee beans, (b) selection of microbiological spoilage of Lactic Acid Bacteria (LAB), and (c) monitoring of potable water. In each case, the novel EN was able to identify the spoiled product by means of the alterations in the pattern of volatile organic compounds (VOCs), reconstructed by principal component analysis (PCA) of the sensor responses. The achieved results strongly encourage the use of EN in industrial laboratories. Finally, recent trends and future directions are illustrated.

1. Introduction

Aroma is one of the most significant parameters of foods from the sensory point of view. The characteristic flavour of VOCs, so called fingerprint, may offer information about safety and quality of food, performing sometimes as an indicator of process mistakes as well [1].

Indeed, some volatile compounds can be originated from biochemical processes of food, as a consequence of technological food chain or product storage.

Unwanted smell, so-called off-flavour, may involve substances originating from the metabolism of spoilage microorganisms, bacteria, and fungi that adulterate naturally or unintentionally the products before or during its production [2].

In the last decade, electronic noses (EN) have become very popular as monitoring tools in evaluating food quality and safety [3].

In this paper, three important applications of EN in food control were examined, concealing three relevant issues in the food field of food quality and control.

Another main target of this work was to illustrate the broad spectrum of potential uses of sensor technology in this field and to show the potential of the new Nanowire technology.

At SENSOR laboratory, the studies on chemical sensors started at 1988 with the improvement of thin films and then of a new technique for the planning of thin films with an extremely porous structure [4].

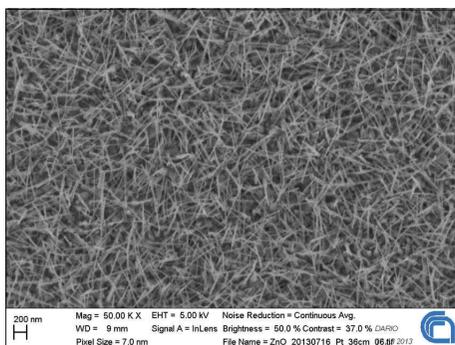


FIGURE 1: ZnO nanowires SEM picture, at 50 k magnification.

In 2001 after the first publications demonstrating the possibility of preparing metal oxide in forms of nanowires and nanobelt, SENSOR demonstrates the ability of metal oxide nanowires in detecting variety of chemical species [5].

It is well known from six decades that metal oxide electrical property depends on the surrounding atmosphere.

Quasi-one-dimensional metal oxide nanostructures (Figure 1) have several advantages with respect to thin and thick film counterparts such as large surface-to-volume ratio, lateral dimensions comparable to the surface charge region, and superior stability when in the single crystal structure [6].

Single crystal nanostructures (Figure 2) of tin oxides have been fabricated and characterized as sensing materials to be implemented in an electronic nose.

These nanowires exhibit remarkable crystalline quality and a very high length-to-width ratio, resulting in enhanced sensing performances as well as long-term stability for sustained operation [7].

2. Materials and Methods

- (a) All the coffee, produced and consumed, belongs to the genus *Coffea* that focuses principally two species: *C. arabica* and *C. canephora*. Those species generally mature in the equatorial zone, where the environment conditions of humidity, temperature, wind, rains, and altitude permit the harvest of many different varieties, each one with particular requirements.

Most of the producers are developing countries, so the storage conditions and the methods used for harvesting and shipping of green coffee beans depend on the production country.

For this reason, it can be easily contaminated by mould throughout the food chain.

Usually the contamination appears due to moulds belonging to the genus *Aspergillus*.

The selection of the raw material occurs in the early stages of the processing chain by visual inspection. Parameters that determinate the quality of green coffee beans are shape, colour, and size.

Frequently the raw material is already contaminated when this selection occurs and thus the detection of the contamination became very difficult.

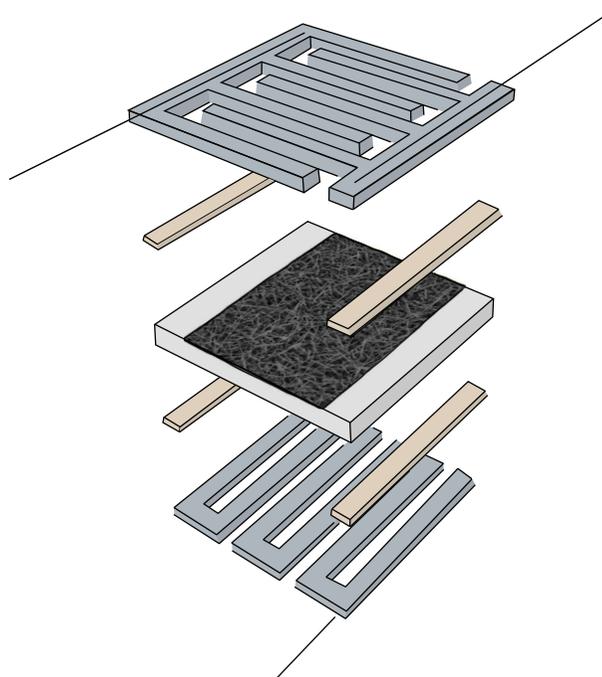


FIGURE 2: Sketch of the conductometric device. Alumina substrate is white colored in the middle, TiW pads are in brown, and platinum heater and contacts are in metallic gray.

Rose-Bengal Chloramphenicol Agar (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was used, as a selective medium for the enumeration of yeast and moulds from a wide variety of distinctive food matrix.

The Chloramphenicol works as suppressor of bacteria growth, although the Rose-Bengal acts as a limiting agent of the mould growth. In order to assist the enumeration of small colonies, the media have neutral pH. The same kind of medium was used to perform all the analysis.

In the case of GC-MS-SPME and EN analysis, 5 mL of medium was placed into sterile 20 mL chromatographic vial and left to solidify.

All the samples, for microbiological analysis, GC-MS-SPME and EN were prepared on the first day of the analysis, taking this day as zero-time. Once prepared, all the samples were stored and incubated under the same conditions during all the duration of analysis, namely, 28°C for a total of 7 days.

During the 7 days of the experiment, analysis was done at zero-time, inoculation day, at T3 (3 days after the inoculation), at T4 (4 days after the inoculation), at T6 (6 days after the inoculation), and at T7 (7 days after the inoculation).

For each day of GC-MS analysis, 10 vials of every type of samples were prepared (control, Honduras, Indonesia, and India): one of each type was used for the GC-MS-SPME analysis, while the other 36 for the EN analysis.

For each day of analysis, the vials were crimped and incubated in an oven thermostatically regulated at 40°C for 15 minutes, to create the headspace equilibrium.

In order to extract the volatile compound from the samples, a DVB/carboxen/PDMS stable flex (50/30 μm) (Supelco Co., Bellefonte, PA, USA) SPME fiber was used.

To furnish the adsorption of volatile compounds, the SPME fiber was exposed to the headspace of the vials for 15 minutes at room temperature. For desorption of the compounds, the fiber was placed in the injector of the heated GC for 6 min.

The ramping of temperature in the column was performed in the following way: 60°C for 2 min to 100°C at 5°C/min, followed by a rise from 100°C to 240°C at 5°C/min and then this temperature was kept for 5 min. Chromatographic analysis was accomplished using a HP 6890 series GC system, 5973 mass selective detector with a DB-WAX capillary column. The injection was verified in splitless mode at 240°C using helium as gas carrier with a setting flow of 1.5 mL/min.

For the electronic nose the sample headspace (4 mL) was then extracted from the vial in static headspace path and injected into the carried flow (speed 4 mL/min) through a properly modified gas chromatography injector (with the connection tube to the EN kept at 40°C to prevent any condensation).

A synthetic chromatographic air with a continuous flow rate of 10 mL/min was used to recover the sensor baseline, resulting in a recovery time of 28 min.

- (b) In the case of LAB analysis, the samples were treated using spoilage lactic acid microflora isolated from the chicken meat. The used procedure for sampling method was conducted as it is described below. Under sterile conditions, 10 g of chopped chicken meat was placed in a stomacher bag with 90 mL of sterile physiological solution and shaken off in Lab Blender Stomacher 400 (Type BA 7021 Seward, London) for 1 min at normal speed (200 paddles/min).

One mL of the supernatant was inoculated in Man, Rogosa and Sharpe Agar medium (MRSA) (OXOID) [8] Petri dish following the inclusion method. Once the first layer was solid, a second layer was added in order to create the microaerobic environment conditions for the LAB growth. MRSA medium was considered to be supporting the growth of lactobacilli. The plates, inside jar with the gas generating kit (OXOID), were incubated during 48 hours at 30°C.

Then, the 48-hour 3 colonies were randomly picked up and inoculated in a MRS liquid tube in order to obtain liquid cultures. The 3 typologies of tubes were incubated for 48 hours at 30°C.

The samples for EN and GC-MS analysis were prepared using the same procedure. Sterilized chromatographic vials (20 mL) containing 2 mL of MRSA media were inoculated independently with 100 µL of the number 3 of McFarland standards of the 3 kinds of cultures prepared before. These standards are used as a reference to adjust the turbidity of bacterial suspensions in order to have a number of bacteria within a given range. Number 3 of McFarland standard matches a bacterial concentration of 9×10^8 CFU/mL.

Analysis with EN and GC-MS was done at 0 time, inoculation day, and 24 hours later a second cycle of GC-MS was performed to make a control of the head space changes at the end of the EN analysis.

- (c) Regarding the incidence of coliforms, an aliquot of water from wc and a well was dispersed in 2 Petri dishes with Violet Red Bile Agar (VRBA) (OXOID) [9]. VRBA is a selective medium used for the detection and enumeration of coliform bacteria in water and other food dairy products. The goal was to isolate the single colonies that were used later to inoculate liquid tubes of Brilliant Green Bile medium (OXOID) [10] to obtain pure liquid cultures. Brilliant Green Bile medium is a modification of MacConkey's liquid medium for the isolation of Enterobacteriaceae and has been formulated to obtain maximum recovery of bacteria of the coli-aerogenes group, while inhibiting most gram-positive bacteria.

For the 2 kinds of samples the followed procedure was the same. Once the Petri dishes were inoculated, they were conserved at room temperature for 2 days. Then, single colonies were selected and inoculated in liquid tubes of Brilliant Green Bile media and incubated for 24 hours at the optimal growth temperature for coliforms 35°C.

After 24 hours, the turbidity of the tubes was evident, and it was adjusted (diluted, using sterile Brilliant Green Bile medium) until the turbidity was the same as the number 3 of the McFarland standards.

Samples GC-MS-SPME and EN were prepared on the first day of the analysis taking this day like 0 time. Once prepared, all the samples were stored and incubate under the same conditions during all the lasting of analysis, namely, 35°C for a total of 24 hours.

The analyses were done the same day of inoculation, and a second cycle of GC-MS was made 24 hours later. In this case, the samples were not crimped and the ensemble was cover with aluminium foil in order to keep vial and cap combined to preserve the sterility inside and, at the same time, afford the aerobic condition for the bacterial growth.

Principal component analysis (PCA) performed explorative data analysis. Data were processed by EDA software, at-home-written software developed in MATLAB at SENSOR laboratory [11].

Exploratory data analysis (EDA) is a fundamental step in the data analysis cycle (the cycle consists of data acquisition, data preprocessing, exploratory data analysis, and classification).

The aims of explorative analysis are as follows: maximize insight into a data set, uncover underlying structure, extract important features, and detect outliers. The most valuable outcome of EDA is to check for prior assumptions and determine optimal experimental settings.

3. Results and Discussion

- (a) After 7 days of incubation (classical microbiological analysis) the differences among the inoculated plates were manifested. Each sample corresponds to one of the 3 different provenances of coffee selected for the experiments. It's perfectly shown the differences in number and typology of colonies between the samples.

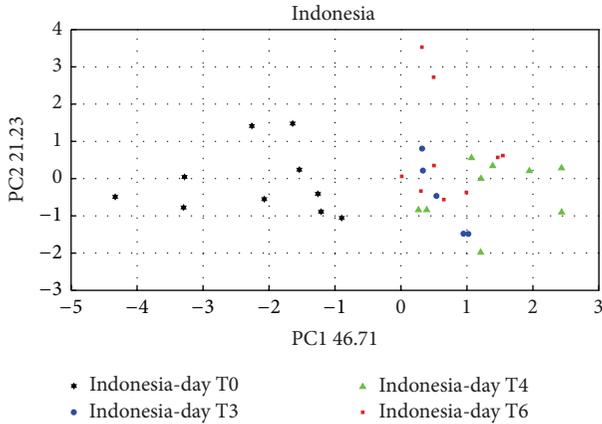


FIGURE 3: PCA score plot about Indonesia green coffee beans during 6 days of analysis (T0 to T6).

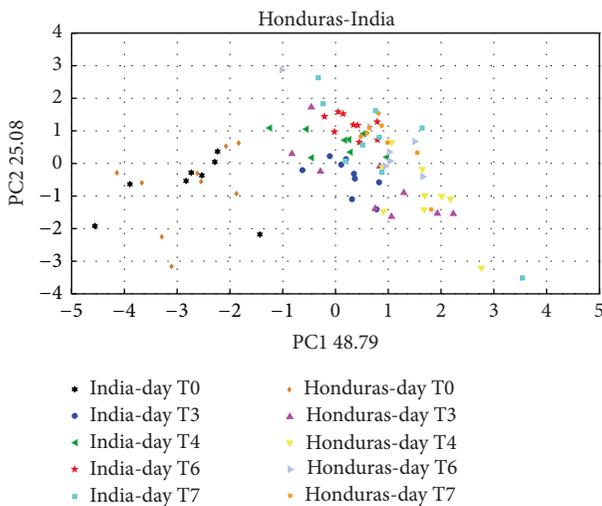


FIGURE 4: PCA score plot about the comparison of two different origins, Honduras and India green coffee beans (T0 to T7).

These results suggest that coffee from different provenances have qualitative and quantitative difference in indigenous contamination.

Results obtained with GC-MS showed to be in perfect correlation with those obtained with the other two techniques.

These differences are both qualitative and quantitative, showing in all cases, except the control, compounds that corresponded with the metabolites produced by moulds during their growth.

In particular, it can be highlighted the formation of carbon dioxide, ethanol, and compounds belonging to chemical indole group [12].

In the figures (Figures 3 and 4) are shown the data related to Indonesia, Honduras, and India, from day T0 (day of the sample preparation) to days T6 and T7 (six and seven days after sample preparation).

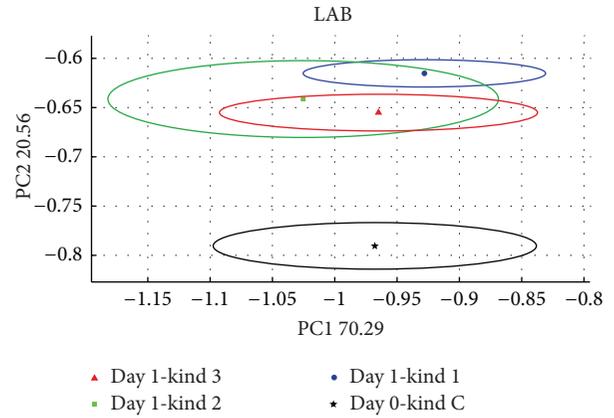


FIGURE 5: PCA score plot about control in a separate cluster and 3 different kinds of LAB. In the blue circle the kind 1, in the red circle the kind 3, in green circle the kind 2, and in black circle in the bottom the control sample.

It is clearly visible a separation in the PC1 axis among the days of analysis, showing development of the growth of the moulds that in both case has more statistical significance.

In the previous literature [12], all the results were referred to an array of 6 MOX thin film sensors that provide a real individualization of the samples only at day T6.

The new array composed of 4 MOX [7] thin film sensors and other 2 made up with MOX nanowire technology; the differences between samples are evident already at T3, halving the response time and hence increasing the instrument threshold.

(b) In Figure 5 are showed the result from the PCA analysis of LAB. It can be separate 2 principal clusters. One denotes to the control (black circle), and the second one is formed for 3 typologies, samples belonging to different colonies.

The creation of cluster like this one can be due also because the VOCs that the EN is able to detect were present in the total pathway of the LAB and to the similarity of the colonies because of the same provenience of the samples, all them indigenous contaminated of the chicken meat.

Regarding the kind 1 (blue circles), samples belonging to colony 1 it worth to think that can be bacteria from the same group but perhaps to different species so it will explain that some of the samples start to move away from the general cluster.

The result obtained in LAB, with GC-MS (Figure 6), case seems to be in perfect correlation with those obtained with the EN. Actually, there are evident qualitative and quantitative differences within the components of samples of the same group.

(c) In the case of PCA (Figure 7) from the EN analysis of coliforms, a separate cluster formed by the samples of the bacteria isolate from the well and those belonging to wc can be observed. Very interesting results came out concerning the samples proceeded from the isolate of the wc. In this case the different measures

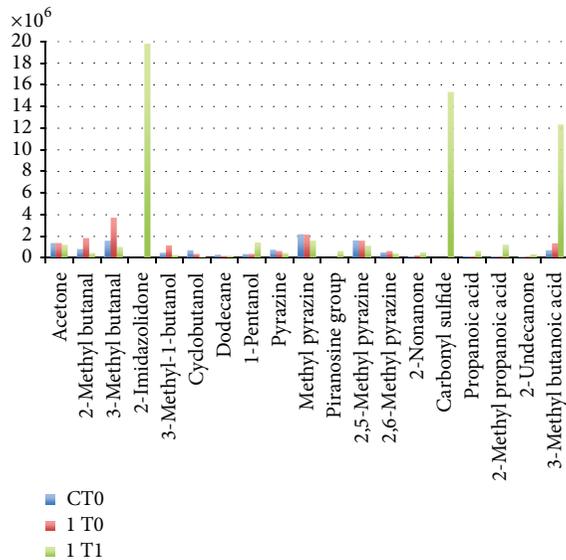


FIGURE 6: Histogram obtained from GC-MS, with SPME techniques about VOCs produced by control and one kind of LAB.

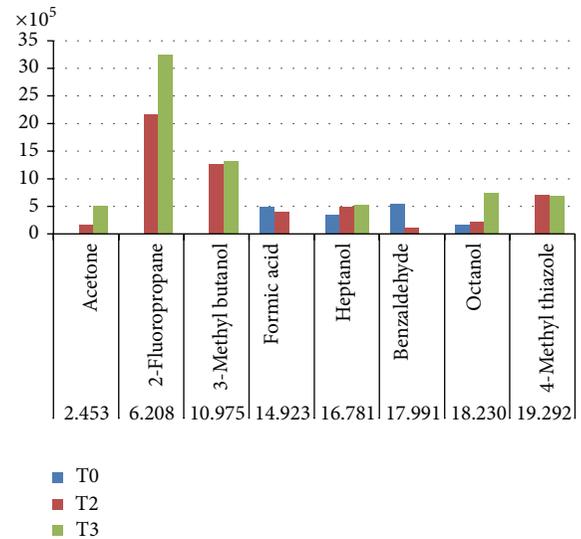


FIGURE 8: Histogram obtained from GC-MS, with SPME techniques about VOCs produced by wc samples (T0 to T2).

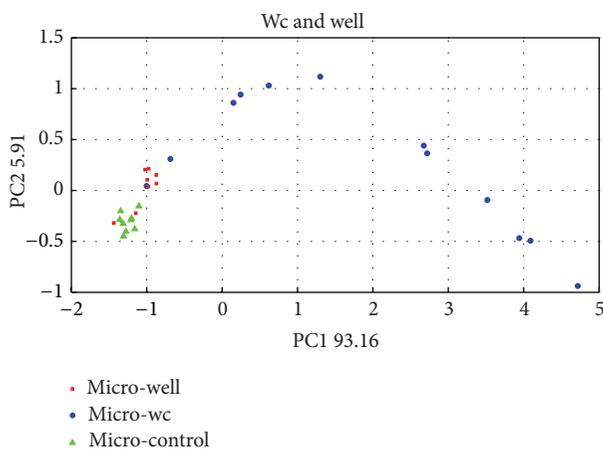


FIGURE 7: PCA score plot analysis of microorganism isolated from wc and well (pozzo).

form a curve perfectly correlated with the time in which the measure was done, showing a kind of curve from bacterial growth. This growth correspond to 20 hours of EN analysis and the curve showed by the PCA analysis can be compared with the first steps of the typical logarithmic growth curve of this species of microorganism.

In the case of coliforms, it can be observed, in the results obtained with GC-MS (Figure 8), an increase of the acid compounds like benzoic, hexanoic acid, 3 methyl butanol, and also some compounds with an alcohol group like nonanol and acetone.

In some cases, EN is able to detect differences between samples while the GC-MS is not able to reveal a production of metabolites created by the growing bacteria. That is owing to the consumption, by the bacteria, of some metabolites present

in the medium, making the EN respond in different way and revealing differences between the samples.

4. Conclusions

In this work, some significant applications of an electronic nose, based partially on metal oxide nanowires technology, to microbiological food quality control have been review. Literature review has been accompanied by some significant case studies previously presented in this field by the same authors, in order to provide the reader with an enhanced perceptiveness of the EN application.

All the described case studies showed promising results, thus confirming that our EN could represent a rapid mean for controlling and improving the microbiological quality of food.

Keeping in mind advantages and limitations, EN does not allow replacing human panels or analytical techniques yet. Their ability to smell odours rather than detecting and quantifying specific volatiles (VOCs) can still be improved. However, they can be used in parallel to those techniques, or ever considered as valuable alternative. Current methodology involves conventional technique such as classical microbiology, visual techniques, or molecular techniques and requires in most of cases a big amount of time, not always available.

This work attests that the electronic nose, once trained, is a potential and useful (rapid and economic) tool for the early detection of microbial grows. A kind of sensor technology like a novel EN provides a faster response of the detection of contaminations in food matrix than the conventional techniques (also compared with the commercial EN equipped only with traditional MOX sensors).

In some cases the novel EN is able to anticipate the detection in a few days with respect to the commercial one [12]. It is possible thanks to the dimensional structure of

the sensors that give more surfaces for contact with the VOC's map.

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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

Efficacy of Three Light Technologies for Reducing Microbial Populations in Liquid Suspensions

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The aim of the current study was to evaluate the effectiveness of three nonthermal light technologies (NUV-Vis, continuous UV, and HILP) on their ability to inactivate *Escherichia coli* K12 and *Listeria innocua*. *E. coli* K12 was selected as a representative microorganism for the enterohaemorrhagic foodborne pathogen *E. coli* O157:H7 and *L. innocua* as a surrogate microorganism for the common foodborne pathogen *Listeria monocytogenes*, respectively. The liquid matrix used for the disinfection experiments was a liquid matrix (MRD solution). The results of the present study show that the HILP treatment inactivated both *E. coli* and *L. innocua* more rapidly and effectively than either continuous UV-C or NUV-vis treatment. With HILP at 2.5 cm from the lamp, *E. coli* and *L. innocua* populations were reduced by 3.07 and 3.77 log₁₀ CFU/mL, respectively, after a 5 sec treatment time, and were shown to be below the limit of detection (<0.22 log₁₀ CFU/mL) following 30 sec exposure to HILP (106.2 J/cm²). These studies demonstrate the bactericidal efficacy of alternative nonthermal light technologies and their potential as decontamination strategies in the food industry.

1. Introduction

In recent years, nonthermal technologies have shown potential as alternatives to conventional pasteurization, with scope for inactivating pathogens and spoilage microorganisms without any of the adverse effects on product quality associated with thermal treatments such as reduced nutritional value or altered sensory attributes [1, 2].

Some pathogenic bacteria such as *Listeria monocytogenes* and other psychrotrophic bacteria can grow at low temperatures, threatening public health and shortening the shelf life of raw foods [3]. Many outbreaks associated with fresh ready-to-eat produce have been reported previously with *E. coli*, *Listeria*, and *Salmonella* identified as implicated pathogens [3–8].

Currently fresh produce, fruit, and vegetables, are washed with aqueous sanitizers such as chlorine, hydrogen peroxide, and trisodium phosphate in order to reduce the microbial

load of fresh produce. However, the use of aqueous sanitizers alone has not been successful in controlling foodborne pathogens [9] and treatment of produce with chlorine has adverse effects, such as formation of trihalomethanes [10, 11]. Organic acids, mainly citric, lactic, and acetic acid, which are in GRAS (Generally Recognized As Safe) status, have been also used as disinfectants because of their bactericidal activity [12]. Hydrogen peroxide (H₂O₂), also referred to as hydrogen dioxide, has also been used as bleaching agent due to its strong oxidizing power [12]. As a result there is a need for the development of additional effective hurdles for these raw foods which can eliminate or significantly reduce microbial contamination, be environmentally friendly while not impacting on the quality of foods [4]. A range of nonthermal technologies (Ultrasound, UV-C, Ozone, and HHP) have already been successfully implemented on a number of ready-to-eat fruits and vegetables [11, 13–17].

NUV-vis light 395 ± 5 nm is a safe, non-UV based decontamination technology which is thought to act by stimulating endogenous microbial porphyrin molecules to produce oxidizing reactive oxygen species (ROS), predominantly singlet oxygen (1O_2) that damages cells leading to microbial death [18–21]. Exposure of microorganisms to visible light particularly at wavelengths of 405 nm, has been shown to be effective in inactivating a range of bacteria, including Gram- positive and Gram-negative bacterial species and antibiotic-resistant microorganisms such as Methicillin-resistant *Staphylococcus aureus*, and its use has been suggested for a range of decontamination applications [22–26].

The inactivation mechanism of UV light is the formation of photoproducts in the DNA of target microorganisms. Of these photoproducts, the most important is the pyrimidine dimer which forms between adjacent pyrimidine molecules on the same strand of DNA and can interrupt both DNA transcription and translation [27]. The DNA damage inflicted by UV-C radiation leads to cell death by altering the microbial DNA through dimer formation between neighbouring pyrimidine nucleoside bases in the same DNA strand [28, 29].

High-intensity light pulses (HILP) is a nonthermal technology which uses short (100–400 μ s) high-power pulses of broad-spectrum (200–1100 nm) and has been used to inactivate bacteria (vegetative cells and spores), yeasts, moulds, and even viruses [30, 31]. The mode of action of HILP on microorganisms is likely the photochemical action of the UV-C part of the light spectrum that causes thymine dimerization in the DNA chain preventing replication and ultimately leading to cell death [2, 32–34]. Microbial inactivation using HILP has gained attention in recent years due to lower energy consumption compared to conventional thermal processes [35]. Depending on the energy delivered through each flash, the distance between the lamps and the contaminated matrix, the targeted microorganism, and even the nature of the contaminated matrix itself, HILP has been reported to result in a 0.5 to 8 \log_{10} CFU/mL bacterial reduction [36]. The germicidal action of HILP has been also attributed to the localized elevated temperature due to the UVs and IR radiations leading to bacterial disruption [33, 37–40].

The objective of the present work was to evaluate the effectiveness of three nonthermal light technologies (NUV-vis, Continuous UV, and HILP) to reduce microbial populations in a liquid matrix. Different treatment intensities and times were selected in order to investigate the inactivation capacity of each light technology on one Gram-negative (*E. coli* K₁₂) and one Gram- positive bacteria (*L. innocua* NCTC 11288). *E. coli* K12 and *L. innocua* were selected as surrogate organisms for *Escherichia coli* O157:H7 and *Listeria monocytogenes*, respectively [2]. To the authors' knowledge, this is the first paper where all these three nonthermal light technologies were compared for their ability to inactivate possible foodborne pathogens.

2. Material and Methods

2.1. Microorganisms and Culture Preparation. Experiments were conducted using *E. coli* K₁₂ (DSM 1607) and *L. innocua*

(NCTC 11288). The strains were maintained at 4°C on Tryptone Soya Agar, TSA (Oxoid, Hampshire, UK). For inoculation of the model solutions, cultures of *E. coli* or *L. innocua* grown overnight at 37°C in Tryptone Soya Broth, TSB (Oxoid), were used. The 24 h cultures were then centrifuged for 10 min at 30,000 \times g and the resulting pellets were washed and centrifuged twice in Maximum Recovery Diluent (MRD, Oxoid) before being mixed together by resuspending in a final volume of 10 mL MRD. This resulted in mixed culture cell suspensions of $\sim 10^8$ colony forming units per milliliter (CFU/mL). The suspensions containing both *E. coli* and *L. innocua* inoculates were assessed for susceptibility to three light technologies in a liquid matrix (MRD). Mixed pure cultures (in MRD) were prepared as described previously. Samples (10 mL) were then placed into Petri dishes (50 mm diameter). They were then positioned at different distances from the lamp source. After removal of covers, Petri dishes containing the MRD solutions were subjected to different light doses ranging from 0.18 to 106.2 J/cm².

2.2. UV Equipment

2.2.1. NUV-Vis Light Unit. The NUV-vis light was produced by a light-emitting diode (LED) array (OD-2049) (Opto Diode Corp, sourced from AP Technologies, Bath, UK) with a central wavelength of 395 ± 5 nm, a bandwidth of 12 nm full-width at half maximum (FWHM), and a half intensity beam angle of 30° [40]. The irradiance ($J \cdot cm^{-2}$) of light emitted from the LED unit was measured using a UV-VIS Radiometer (model no. RM12, Dr. Gröbel UV Elektronik, GmbH, Ettlington, Germany) fitted with a RM UV-A sensor (part no. 811030, Dr. Gröbel UV Elektronik). Distances of 3, 12, and 23 cm from the light source were chosen for treatments. The corresponding energy intensities and time needed to achieve them are presented in Table 1. These distances represented the most extreme to the least extreme treatments according to the study of Haughton et al. [41]. Construction of the LED unit was as previously described [41]. Sample temperatures were measured during the treatment using a K-type thermocouple attached to a Grant Data Logger (Squirrel 2040; Grant Instruments) to ensure that the maximum temperature reached was nonlethal to the bacteria under the treatment times investigated (<50°C) (Figure 6).

2.2.2. Continuous UV Equipment. The UV unit was a custom-made unit with intimal dimensions (length \times width \times height) of 790 \times 390 \times 345 mm and consisting of four 95-W bulbs (Baro Applied Technology Limited, Athens, Greece) 500 mm in length. The UV dose (D) was calculated by using the following equation:

$$D : I_{254\text{ nm}} * t, \quad (1)$$

where D is the dose (J/cm²), $I_{254\text{ nm}}$ is the dosage rate, and t is the retention time (in seconds). The UV dosages (J/cm²) were varied by altering the distance of the sample (6.5, 17, and 28.5 cm) from the light source and by changing the treatment time (Table 1). Sample temperatures were measured during the treatment using a K-type thermocouple attached to a

TABLE 1: Calculated exposure time (sec) of nonthermal light technologies at selected distances from the light source.

Distance from light source (cm)		Dose per treatment (J/cm ²)											
		0.18	0.36	0.72	1.44	2.832	6	17.7	27	36	54	106.2	
3 12 23 6.5 17 28.5 2.5 8 11.5 14	NUV-VIS	6	11	22	45	88	186	548	836	1115	*	*	
		28	55	110	221	435	*	*	*	*	*	*	
		149	298	595	1190	2341	*	*	*	*	*	*	
	UV	30	60	120	240	472	*	*	*	*	*	*	
		36	72	144	288	566	*	*	*	*	*	*	
		45	90	180	360	708	*	*	*	*	*	*	
	HILP	NT	NT	NT	NT	0.8	NT	5	NT	NT	NT	NT	30
		0.1	0.2	0.4	0.8	NT	NT	NT	NT	NT	NT	30	NT
		NT	0.3	0.6	NT	NT	5	NT	NT	NT	30	NT	NT
0.2		0.4	0.8	NT	NT	NT	NT	NT	30	NT	NT	NT	

NUV-Vis: near UV-vis light; UV: ultraviolet light; HILP: high-intensity light pulses.

(*) Samples that are not analyzed due to high temperature, NT: not tested samples.

Distance from light source (cm).

HILP was applied in pulses 360 μ s duration at a frequency of 3 Hz.

Grant Data Logger (Squirrel 2040; Grant Instruments) to ensure that the maximum temperature reached was non-lethal to the bacteria under the treatment times investigated (<50°C) (Figure 7).

2.2.3. HILP (High-Intensity Light Pulses) Unit. The HILP unit was a benchtop SteriPulse-XL system (Xenon, USA). The system comprised a high-energy pulsed ultraviolet-visible flash lamp (Type C, 190 nm spectral cut-off point) delivering a maximum of 1.27 J/cm². The pulse width produced was 360 μ s at a fixed pulse rate of 3 Hz. The pulse energy delivered to the sample varied depending on its distance from the quartz window within the HILP chamber. The HILP dose of treatments applied in the present study was calculated in accordance with the manufacturer's instructions [42]. Distances of 2.5, 8, 11.5, and 14 cm were selected for treatments, in order to achieve a wide spectrum of dosages varying between 0.18 and 106.2 J/cm². The corresponding dosages and time needed to achieve them are presented in Table 1. During HILP treatment, samples were placed in an iced bath to minimize heating. Sample temperatures were measured during the treatment using a K-type thermocouple attached to a Grant Data Logger (Squirrel 2040; Grant Instruments, Cambridge, UK) to ensure that the maximum temperature reached was nonlethal to the bacteria under the treatment times investigated (<50°C) (Figure 8).

2.3. Microbiological Analysis. After treatment of liquid samples, the contents of each Petri dish were transferred to sterile containers. Tenfold dilution series were prepared in MRD and 0.1 mL of each dilution was pour plated in duplicate using TBX (CM0945, Oxoid) for *E. coli* and *Listeria Selective Agar* (Oxford formulation, CM0856, Oxoid) for *L. innocua*. The plates were incubated at 44°C and 37°C for 24 and 48 h, respectively. Mean counts for each treatment were calculated and converted to log₁₀ CFU/mL values with

results for surviving numbers of microorganisms in MRD expressed per mL (CFU/mL). The plates were then used to enumerate viable cells in untreated controls and in samples following processing. The survival of bacterial cells following illumination was monitored by counting their viable number after exposure of the suspended bacteria to light. Bacterial cultures grown under the same conditions but without light exposure served as controls. The results were expressed as the logarithmic reduction (log₁₀N/N₀), where N₀ is the initial microbial load and N the number remaining after treatment. All experiments were repeated at least three times.

2.4. Statistical Analysis. All experiments were carried out in triplicate. During each experiment two samples were taken at any time to conduct microbial counts. The microbiological data were analyzed in terms of log₁₀(N/N₀), where N is the microorganism load at a given time, and N₀ corresponds to the initial microbial load of untreated samples. The data for inactivation of *E. coli* and *L. innocua* by NUV-vis light, continuous UV and HILP were analyzed for statistical significance using SPSS 21.0 (SPSS Inc., Chicago, USA). Results were compared by an analysis of variance followed by Tukey's pairwise comparison of the means with significance defined at the P < 0.05 level. Moreover, Pearson coefficient was used for measuring correlation between values.

3. Results

3.1. Inactivation Using NUV-Vis 395 ± 5 nm. The inactivation rate of *E. coli* and *L. innocua* was dose dependent. Generally, it was observed that as the distance from the lamp was increased, the time needed for inactivation was longer (Figure 1). Corresponding doses delivered by this method are illustrated in Table 1.

When low dosages were implemented (0.18, 0.36, 0.72, and 1.44 J/cm²), the observed inactivation rates were similar

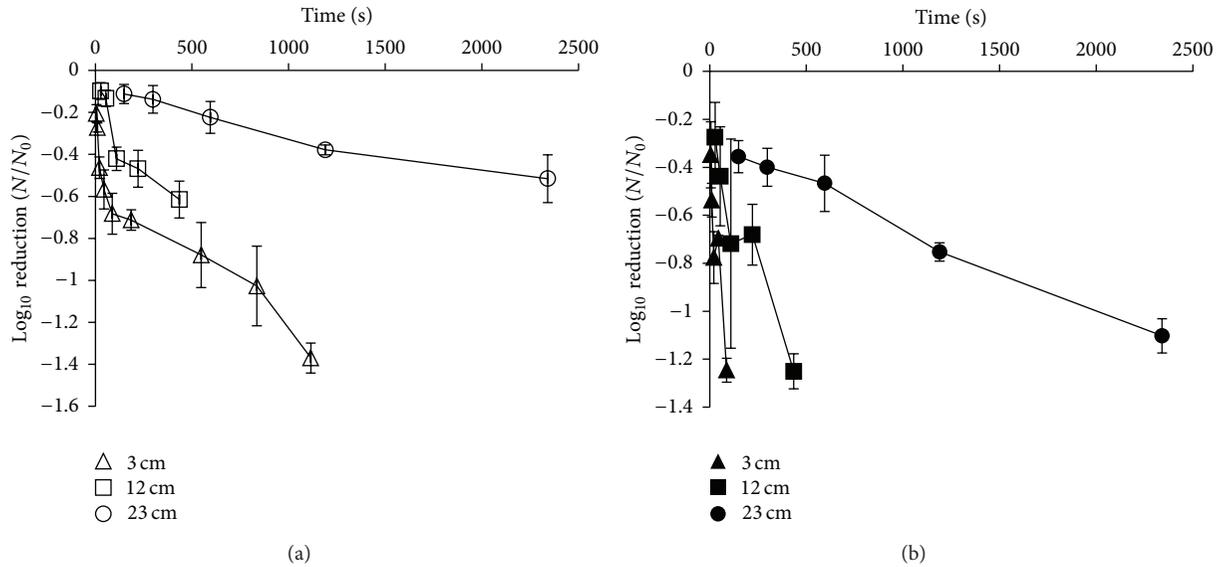


FIGURE 1: Survival curves of *E. coli* suspended in maximum recovery diluent (MRD) placed at 3 cm (Δ), 12 cm (\square), and 23 cm (\circ) and *L. innocua* placed at 3 cm (\blacktriangle), 12 cm (\blacksquare), and 23 cm (\bullet) from the high-intensity near ultraviolet/visible (NUV-vis) 395 ± 5 nm light source (results expressed as mean \log_{10} CFU/mL).

for both *E. coli* and *L. innocua* ($P > 0.05$). However, when a higher dose of 2.832 J/cm^2 was delivered, *L. innocua* exhibited a higher log reduction ($1.25 \log_{10}$ CFU/mL) compared to *E. coli* ($0.68 \log_{10}$ CFU/mL) after 88 sec of treatment ($P < 0.05$), around 2 times the inactivation log of the more resistant bacterium of *E. coli*. Moreover, when high dosages were achieved, the inactivation rates of *L. innocua* remained significantly higher with a maximum average \log_{10} CFU/mL reduction of 2.74 achieved after 1115 sec of treatment, compared to that of *E. coli* where the maximum average log reduction after the same time was $1.37 \log_{10}$ CFU/mL ($P < 0.05$). Moreover, the log reduction achieved for *L. innocua* remained higher than that of *E. coli* after the longest exposure time. At 23 cm from the light source, a higher susceptibility was observed for the *L. innocua* strain, giving a log reduction at the highest dose (2.832 J/cm^2) of $1.10 \log_{10}$ CFU/mL, significantly greater ($P > 0.05$) than the corresponding reduction for *E. coli* ($0.52 \log_{10}$ CFU/mL reduction). It has to be mentioned that temperatures remained below 50°C for all treatments used in the study.

3.2. Inactivation Using Continuous UV Light. There was an increase between dosage and \log_{10} CFU/mL reduction of both microorganisms. Reductions of $2.66 \log_{10}$ CFU/mL and $3.04 \log_{10}$ CFU/mL were achieved for *E. coli* and *L. innocua*, respectively. The highest reductions were achieved at the shortest distance from the lamp (6.5 cm) and at the shortest treatment time (472 sec) (Figure 2) when a dose of 2.832 J/cm^2 was implemented (Table 1). However, the susceptibility of two microorganisms when this light technology was used was not significantly different ($P = 0.749$). Temperatures remained below 50°C for all treatments used in the study.

3.3. Inactivation Using HILP. The measured dosages delivered with this light method and the time needed to achieve them is illustrated in Table 1. In general, increased dosage resulted in the greater reductions for both *E. coli* and *L. innocua*. The least susceptible microorganism was *E. coli* (Figure 3). A dosage of 17.7 J/cm^2 resulted in log reductions of *E. coli* and *L. innocua* populations (3.07 and $3.77 \log_{10}$ CFU/mL, resp.) and were both below the limit of detection ($<0.22 \log_{10}$ CFU/mL) following exposure to HILP at 106.2 J/cm^2 . When a dosage of 54 J/cm^2 was implemented, reductions of 4.81 and $5.56 \log_{10}$ CFU/mL were achieved for *E. coli* and *L. innocua*, respectively. At a dosage of 36 J/cm^2 , a degree of variation was observed between the two tested microorganisms. For example, *E. coli* was reduced by $3.85 \log_{10}$ CFU/mL, whereas *L. innocua* was reduced by $5.30 \log_{10}$ CFU/mL ($P < 0.05$). The susceptibility of two microorganisms when this light technology was used was significantly different ($P < 0.05$). Temperatures did not exceed 50°C for any of the HILP treatments used in the current study.

3.4. Comparisons between Three Light Technologies. The three light technologies were tested for their disinfection capacity on two microorganisms (Figures 4 and 5). At low dosages (0.18 , 0.36 , and 0.72 J/cm^2) the results between the two microorganisms, when the three light technologies were used, were all significant ($P < 0.05$). When 1.44 J/cm^2 was implemented, the \log_{10} (CFU/mL) reduction at NUV-vis light and continuous UV light for both microorganisms was significant ($P < 0.05$), whereas when comparisons with HILP light were done, the differences between the susceptibility of the tested microorganisms did not differ ($P > 0.05$). When 2.832 J/cm^2 was implemented in both

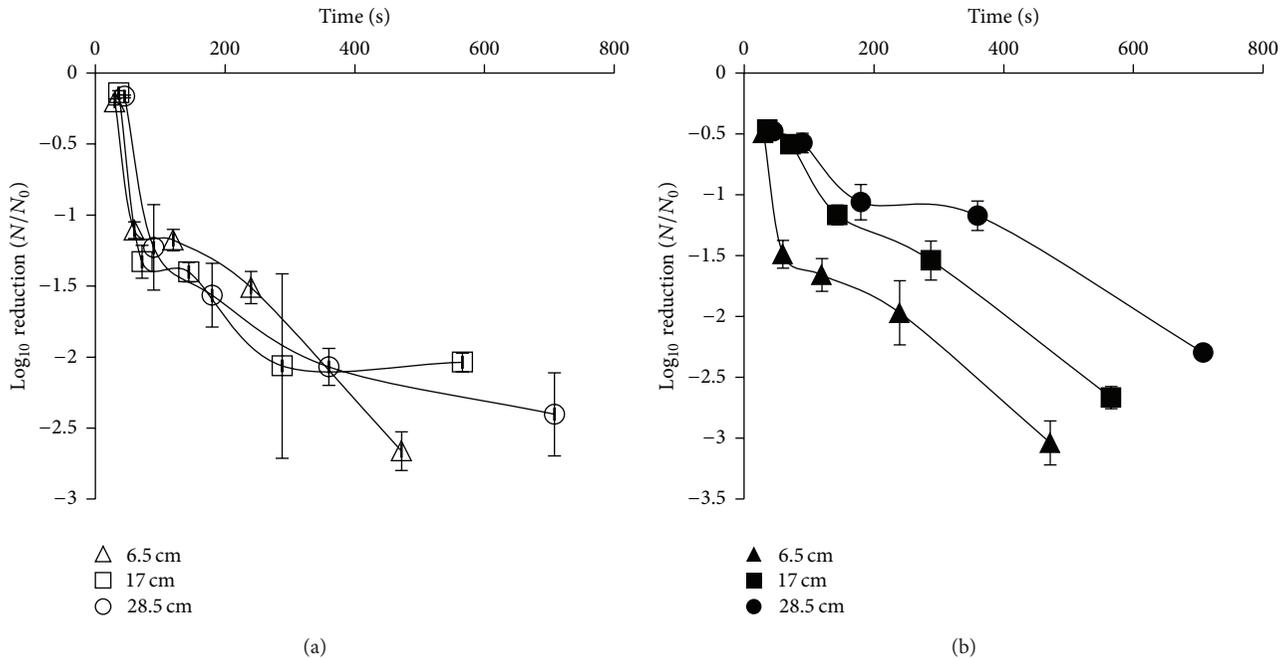


FIGURE 2: Survival curves of *E. coli* suspended in maximum recovery diluent (MRD) placed at 6.5 cm (Δ), 17 cm (□), and 28.5 cm (○) and *L. innocua* placed at 6.5 cm (▲), 17 cm (■), and 28.5 cm (●) from continuous UV light source (results expressed as mean log₁₀ CFU/mL).

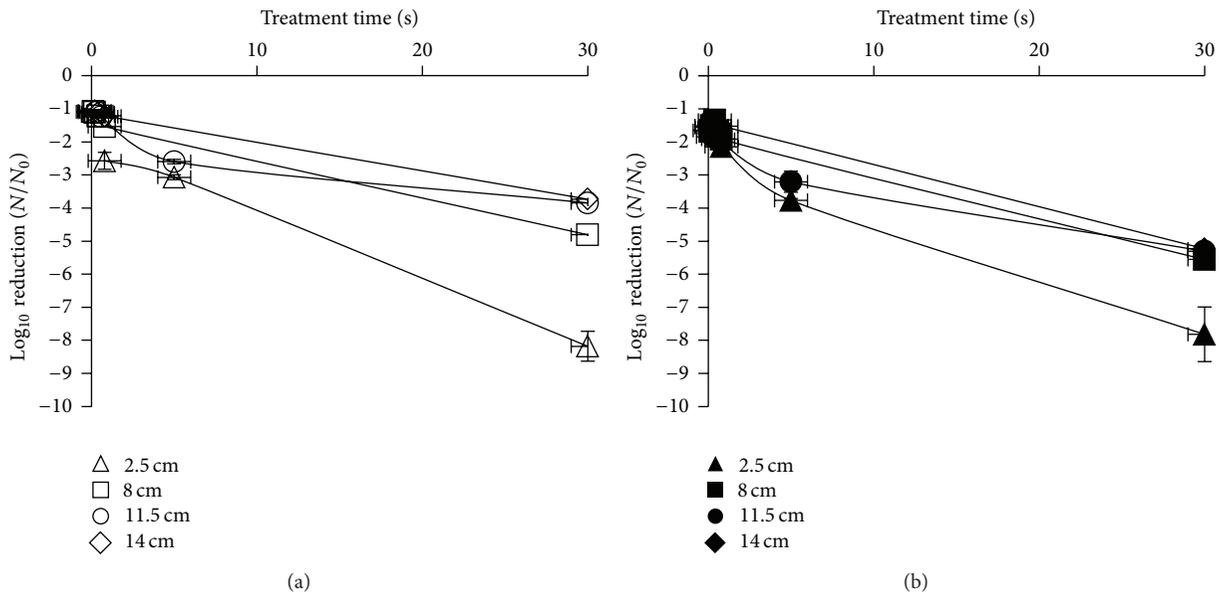


FIGURE 3: Survival curves of *E. coli* suspended in maximum recovery diluent (MRD) placed at 2.5 cm (Δ), 8 cm (□), 11.5 cm (○), and 14 cm (◇) and *L. innocua* placed at 2.5 cm (▲), 8 cm (■), 11.5 cm (●), and 14 cm (◆) from HILP source (results expressed as mean log₁₀ CFU/mL).

continuous UV light technology and HILP, the disinfection efficiency of *E. coli* and *L. innocua* did not differ significantly ($P = 0.306$ and $P = 0.116$, resp.). Finally, at higher dosages, the correlations between log₁₀ (CFU/mL) reduction of NUV-vis and PUV, as the two organisms are concerned, were all significant ($P < 0.05$). Generally, it was observed that in all light technologies (NUV-vis, continuous UV, and HILP) a significant correlation ($P < 0.05$) between doses and microbes log reduction existed (Figures 4 and 5).

4. Discussion

The current study demonstrated that both *E. coli* and *L. innocua* are susceptible to all three light technologies investigated. Previous studies have investigated the lethal effects of high-intensity ultraviolet 405 nm light on *Escherichia*, *Salmonella*, *Shigella*, *Listeria*, and *mycobacteria* as well as on *Saccharomyces cerevisiae*, *Candida albicans*, and spores of *Aspergillus niger* [21, 26]. In a study [43], the inactivation

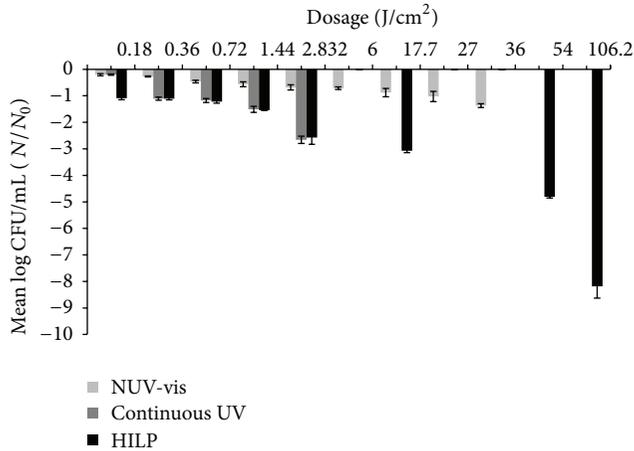


FIGURE 4: Mean \log_{10} CFU/mL *E. coli* on MRD after treatment at the same dosages at shortest distance with 3 different light equipment.

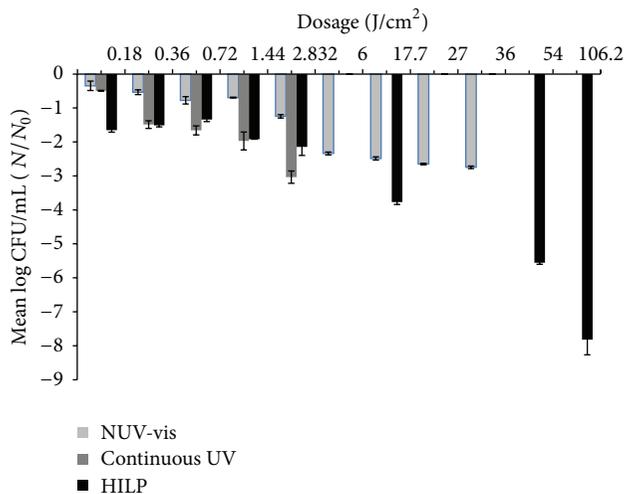


FIGURE 5: Mean \log_{10} CFU/mL *L. innocua* on MRD after treatment at the same dosages at shortest distance with 3 different light equipment.

of *E. coli* and T4 and T7 phages after exposure to HILP was recorded. The evaluation of the effectiveness of HILP for the inactivation of *E. coli* and *L. innocua* in citric acid-sodium phosphate buffer solution was studied from Muñoz et al. [2]. However, the authors are unaware of any studies which directly compared the differences in susceptibility of *E. coli* and *L. innocua* to three different nonthermal light technologies (a NUV-vis light, a continuous UV, and a HILP light). So, the current study set out with two aims: (1) to test the relative susceptibility of two bacteria using three different light techniques; (2) to determine the effectiveness of each light equipment for inactivation of selected types of bacteria when different dosages are implemented.

Although longer treatment times resulted in significant temperature increases in all three light technologies, the dosages that were selected for this study did not result in temperature arise of more than 45°C (Figures 6, 7, and 8).

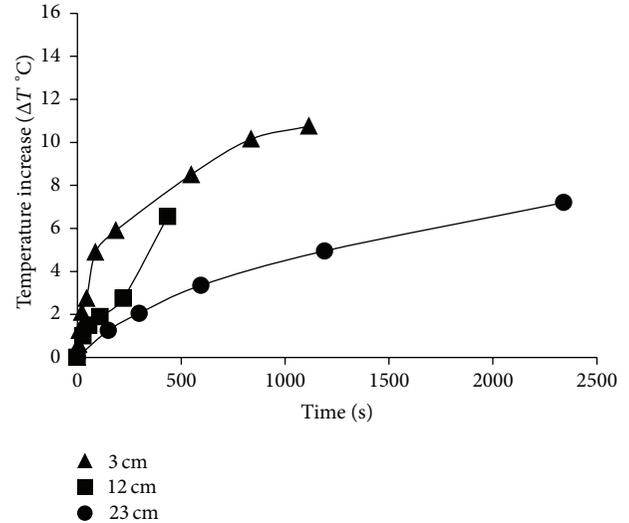


FIGURE 6: Mean Temperature increase ($\Delta T^{\circ}\text{C}$) for NUV-vis light technology at distances.

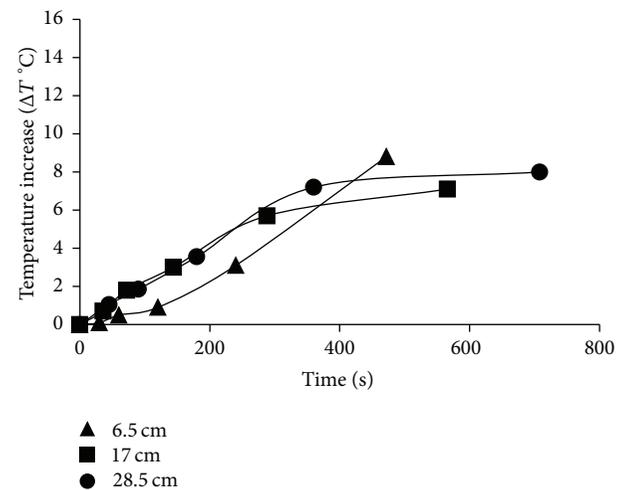


FIGURE 7: Mean Temperature increase ($\Delta T^{\circ}\text{C}$) for UV light technology at distances.

As the mechanism of inactivation by visible light is believed to be through the production of ROS, the susceptibility of both *E. coli* and *L. innocua* to ROS may play an important role in the inactivation of these organisms by NUV-vis light of 405 nm stimulates endogenous microbial porphyrin molecules to produce oxidizing reactive oxygen species (ROS), predominantly singlet oxygen ($^1\text{O}_2$) that damages cells leading to microbial death [20]. Specifically, 405 nm light has been shown to be capable of inactivating a range of predominantly nosocomial pathogens and also Gram-negative food-related pathogens [21, 23].

When NUV-vis light was implemented, *L. innocua* proved to be the most readily inactivated organism compared to *E. coli* ($P < 0.05$). Murdoch et al. [21] found that *L. monocytogenes* was most readily inactivated in suspension, whereas *S. enterica* was most resistant. They concluded that 395 ± 5 nm

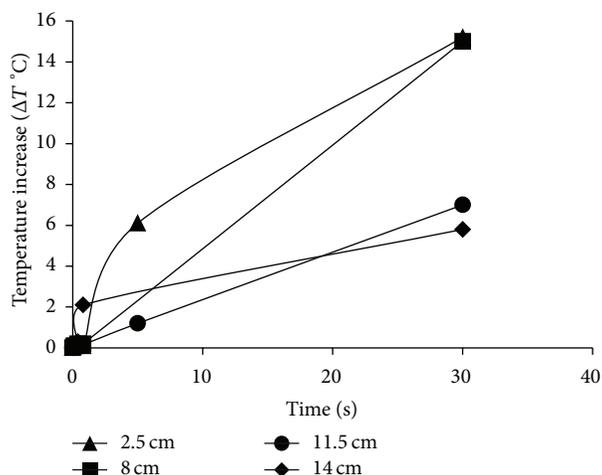


FIGURE 8: Mean Temperature increase ($\Delta T^{\circ}\text{C}$) for HILP light technology at distances.

light inactivates diverse types of bacteria in liquids and on surfaces, in addition to the safety advantages of this visible (non-UV wavelength) light [21]. In addition, it is reported [26] that fungal organisms may be somewhat more resistant to 405 nm light than bacteria. In this study a correlation between dose (J/cm^2) and microbes' inactivation was found. Other studies have reported that Gram-positive species, in general, were more susceptible to 405 nm light inactivation than Gram-negative species, which is generally consistent with the results obtained in the current study [44]. The prokaryotic bacteria also exhibit considerable variability in susceptibility with values, to achieve 5-log_{10} order reductions, as low as $18\text{ J}/\text{cm}^2$ with *Campylobacter jejuni* [45] but most typically around $50\text{--}300\text{ J}/\text{cm}^2$, with Gram-positive species being generally more susceptible than Gram-negatives [44]. Microbial inactivation by 405 nm light exposure has been found to be dose-dependent [21]. In applications where rapid inactivation is desirable, the use of a much higher power light source would significantly reduce the exposure times required for effective treatment. In our study, at the highest dosage ($36\text{ J}/\text{cm}^2$), 1.37 log_{10} CFU/mL reduction was achieved for *E. coli* and a greater log reduction (2.74 log_{10} CFU/mL) was achieved for *L. innocua*. Our results are in accordance with another study [21], where they found that *L. monocytogenes* was completely inactivated at an average dosage of $128\text{ J}/\text{cm}^2$, whereas a 2.18 log_{10} reduction was achieved for *E. coli* at $192\text{ J}/\text{cm}^2$ dosage.

In the present study it was shown that, in order to achieve 2.66 log_{10} CFU/mL reductions for *E. coli* and 3.04 log_{10} CFU/mL for *L. innocua*, respectively, a dosage of $2.832\text{ J}/\text{cm}^2$ with continuous UV equipment was needed. However, the samples were not treated further due to the temperature arise. Our results are not in agreement with other studies [46] where better reductions (7.2 log_{10} CFU/mL reduction and 4.6 log_{10} CFU/mL reduction for *E. coli* and *L. innocua*, respectively, at $1.2\text{ kJ}/\text{cm}^2$) were achieved, perhaps due to different *E. coli* and *L. innocua* strains that were used. UV light creates mutated bases that compromise cell functionality, but

bacteria have developed DNA repair mechanisms to restore DNA structure and functionality [47]. This phenomenon is reflected in the shape of the inactivation curves of our experiment [48].

The killing effects of HILP are caused by the rich and broad-spectrum UV content, the short duration, and the high peak power of the pulsed light produced by the multiplication of the flash power manifold [32, 49]. Other researchers found that a significant reduction of 3.6 log_{10} CFU/mL for *E. coli* K₁₂ and 2.7 log_{10} CFU/mL for *L. innocua* ($P < 0.001$) was achieved with HILP ($3.3\text{ J}/\text{cm}^2$) [2]. Our results are similar to that of study [2] as 2.57 log_{10} CFU/mL reduction for *E. coli* and 2.14 log_{10} CFU/mL reduction for *L. innocua* were achieved when $2.832\text{ J}/\text{cm}^2$ dosage was implemented. To the best of the authors' knowledge three studies referring to the application of high-intensity light pulses in a continuous system [42, 50, 51].

5. Conclusions

The results of the present study show that HILP treatments were more effective for the inactivation of both *E. coli* and *L. innocua*. Furthermore, this technology resulted in more rapid and extensive inactivation than either continuous UV-C and NUV-vis treatments. These observations associated with HILP may be attributable to the comparatively higher penetration depth and emission power compared to continuous UV-C and NUV-vis. Moreover it has a high peak power produced by the multiplication of the flash power manifold, producing a light intensity at least 100 times greater than that of other two light technologies during the same operating time. However, research must be performed in real food matrixes, as it is known that HILP light generates off flavors. It can be concluded that short treatment times for decontamination efficiency would be an important factor related to productivity in food industry. The findings presented here suggest the expansion of the aforementioned light technologies on food decontamination. Thus these alternative nonthermal disinfection light techniques could find potential applications for decontamination in the food industry.

Abbreviations

NUV-vis:	Near ultraviolet/visible light
UV:	Ultraviolet light
HILP:	High-intensity light pulses
ROS:	Reactive oxygen species
MRD:	Maximum recovery diluent.

Conflict of Interests

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

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