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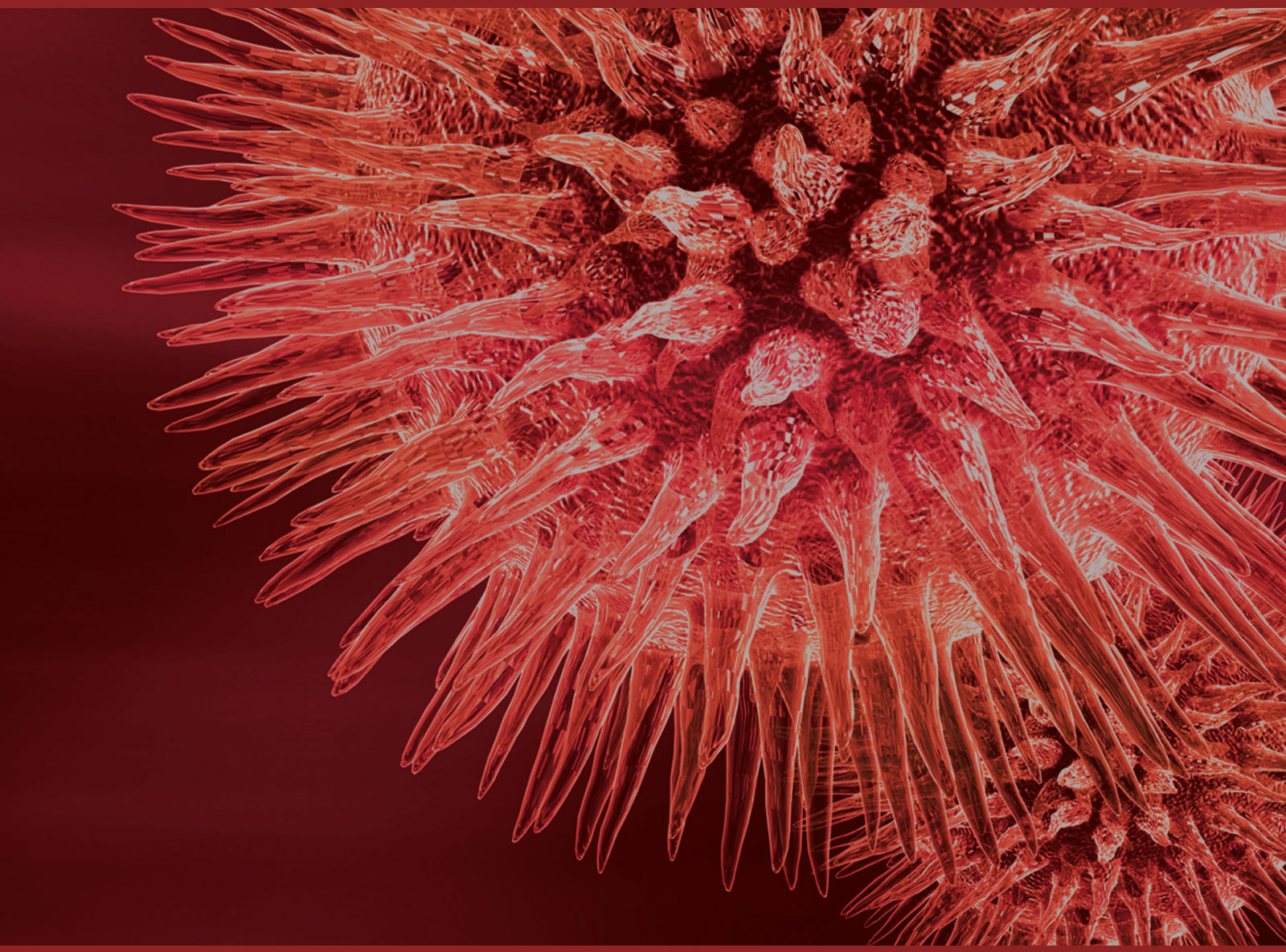
Guest Editors: Petr Kralik, David Rodriguez-Lazaro, Apostolos Vantarakis, and Wim H. M. van der Poel



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Contents

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Research Article

Microbiological Quality of Ready-to-Eat Vegetables Collected in Mexico City: Occurrence of Aerobic-Mesophilic Bacteria, Fecal Coliforms, and Potentially Pathogenic Nontuberculous Mycobacteria

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The aims of this study were to evaluate the microbiological quality and the occurrence of nontuberculous mycobacteria (NTM) in a variety of salads and sprouts from supermarkets and street vendors in Mexico City. Aerobic-mesophilic bacteria (AMB) were present in 100% of RTE-salads samples; 59% of samples were outside guidelines range (>5.17 $\log_{10}$ CFU per g). Although fecal coliforms (FC) were present in 32% of samples, only 8% of them exceeded the permissible limit (100 MPN/g). Regarding the 100 RTE-sprouts, all samples were also positive for AMB and total coliforms (TC) and 69% for FC. Seven NTM species were recovered from 7 salad samples; they included three *M. fortuitum*, two *M. chelonae*, one *M. mucogenicum*, and one *M. sp.* Twelve RTE-sprouts samples harbored NTM, which were identified as *M. porcinum* (five), *M. abscessus* (two), *M. gordonae* (two), *M. mucogenicum* (two), and *M. avium* complex (one). Most RTE-salads and RTE-sprouts had unsatisfactory microbiological quality and some harbored NTM associated with illness. No correlation between the presence of coliforms and NTM was found. Overall, these results suggest that RTE-salads and RTE-sprouts might function as vehicles for NTM transmission in humans; hence, proper handling and treatment before consumption of such products might be recommendable.

1. Introduction

According to the definition given by the FAO and the WHO [1], ready-to-eat (RTE) foods include any comestible that is normally consumed in its raw state. Demand for RTE food has led to an increase in the amount and selection of different products available for the consumers [2]. RTE-salads and RTE-sprouts constitute a suitable and convenient meal for today's lifestyles because they need no cooking or further preparation. As well as being considered low-calorie food, they are rich in fiber and provide a great variety of vitamins, minerals, and other phytochemicals [3]. Their consumption is encouraged in many countries by government health agencies to protect people against a range of illnesses such as cancer and cardiovascular diseases [4]. Therefore, continued increase in the consumption of fresh meals has occurred

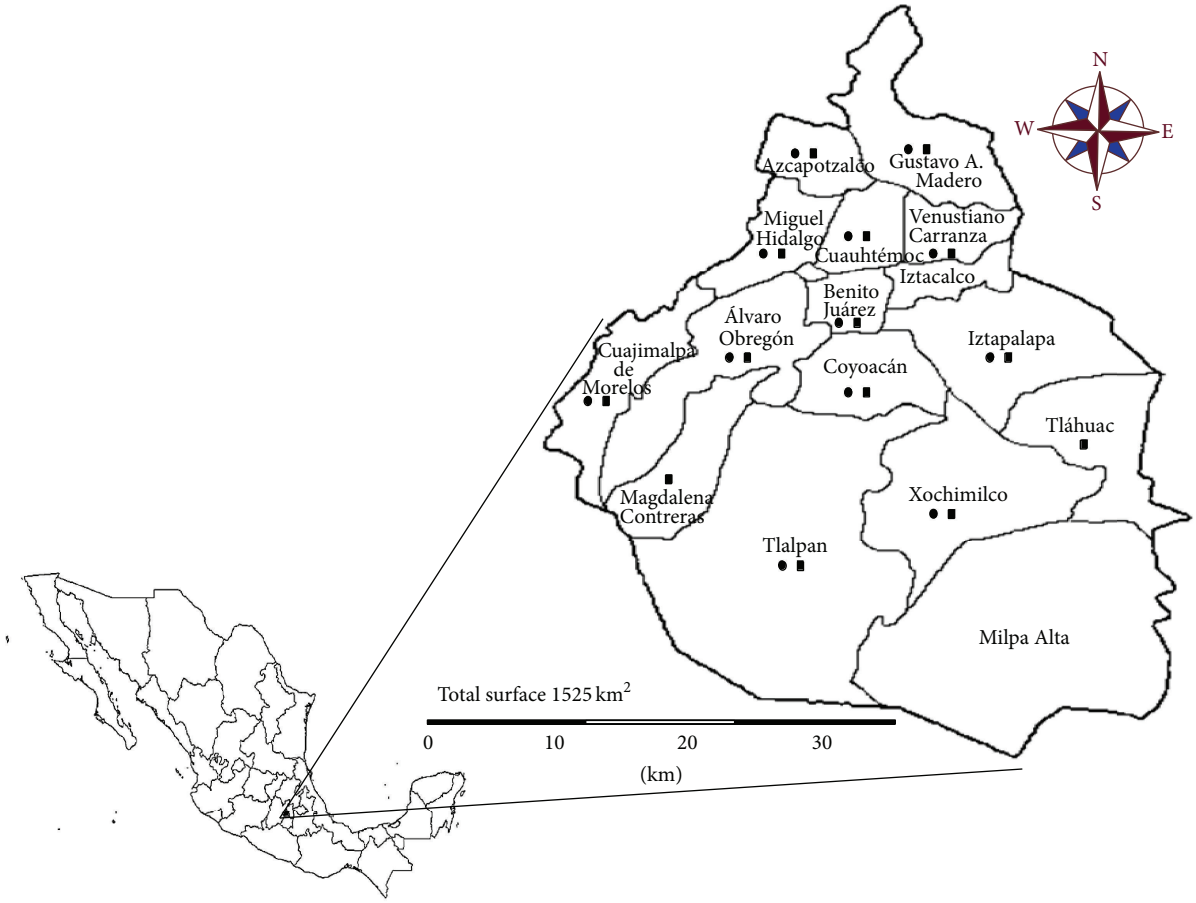

Figure 1: Boroughs of Mexico City where RTE-salads (●) and RTE-sprouts (■) were collected.

as a result of efforts to promote better nutrition in the population [4]. As RTE-salads and RTE-sprouts do not need further preparation before consumption, they could potentially contain pathogens that form part of their microflora, posing a public health problem. Fresh vegetables can become contaminated by pathogens as *Salmonella* at any point during the food production process. During preharvest, contact with contaminated irrigation water, soil, manure, or fecal matter of wild animals may occur. These pathogens can both bind to plant leaves and/or be internalized via the leaves or the endophytic root system [5, 6]. During harvest, asymptomatic human carriers might contaminate the products, and at the postharvest level, products become contaminated by contact with polluted water, other asymptomatic human carriers, or the production process environment. Over the last 30 years there has been at least a 24% of increase in the average amount of fresh vegetable consumed per person in the USA [7]. Moreover, the number of gastroenteritis outbreaks caused by foodborne pathogens after consumption of raw vegetables salads and sprouts has increased worldwide [8–11]. Even though *Salmonella* is the most common cause of disease outbreaks associated with lettuce and sprouts [12–15], there are other pathogens (Shiga toxin, producing *E. coli* O157, Norovirus) that have been described as relevant microbial hazards [16–18]. For example, a large outbreak of hemolytic-uremic syndrome caused by STEC O104:H4 linked to sprouts occurred in Germany [19, 20].

Nontuberculous mycobacteria (NTM) are opportunistic pathogens found in the environment that cause life-threatening infections in humans, other mammals, and birds [21, 22]. The incidence of NTM disease is increasing worldwide [23], in both immunocompetent and immunocompromised subjects [22, 24, 25]. As there is no defining evidence for person-to-person transmission for most of the NTM [24, 25], it is therefore important to establish the sources and routes of NTM transmission, since infections can occur through inhalation, ingestion, gastric reflux, or skin trauma [22, 26]. NTM have been isolated from various kinds of food, and many studies support the hypothesis that food, especially raw or partially cooked products, plays a role as a source of NTM for humans, primarily in countries with similar processing food routes and climates [27–29]. The aims of this study were to evaluate the microbiological quality of RTE-salads and RTE-sprouts collected in Mexico City and to analyze the occurrence of NTM in these samples, in order to determine whether these RTE foods may represent a potential risk for NTM infection for the consumers.

2. Material and Methods

2.1. Area of Study and RTE Food Collection. The selected area of study was Mexico City, a large urban area that, although it has almost 9 million registered inhabitants, during working hours, reaches a population of nearly 25 million. Mexico City is divided into 16 "delegaciones" or boroughs (Figure 1). From January to July 2013 a total of 100 RTE-salads samples of raw vegetables (salads from SPM contained mainly lettuce and spinach accompanied by carrot and purple cabbage, while salads from SVS also contained onion, tomatoes, cucumber, Mexican turnip, mushroom, radish, coriander, cactus, and fruits as strawberry, apple, and mango) were collected from different boroughs (Figure 1): 50 samples from different supermarkets (SPM) and 50 from street-vendor stalls (SVS). Also, 100 RTE-sprouts samples (alfalfa, soybean, broccoli, carrot, radish, onion, amaranth, clover, arugula, lentil, wheat, melon, turnip or combinations of alfalfa and broccoli, alfalfa and clover, alfalfa and onion, alfalfa and soybean, and alfalfa and radish) were purchased (Figure 1), from August 2013 to February 2014: 50 from different SPM and 50 from SVS. Salads and sprouts collected from SPM were purchased in bags provided by a bigger food processing company. Those vegetables collected from SVS were prepared directly by vendors at the selling spot. At the moment of purchase the RTE-salads and RTE-sprouts were packaged in sterile plastic containers and were transported to the laboratory for their analysis within 2 h after collection.

2.2. Microbiological Analysis. According to the FDA [30], 50 grams from each sample was placed in ethylene oxide gas-sterilized polypropylene bags (Whirl-Pak, Nasco, USA) and 450 mL of lactose broth was added in order to achieve a final dilution 1 : 10 (10^{-1}). Samples were homogenized for 2 min in a stomacher (tissue disrupter) and serially diluted (10^{-1} to 10^{-5}); then, these dilutions were used for quantification (CFU/mL) and estimation (MPN/g) of microorganisms. Each sample was tested for the presence of aerobic-mesophilic bacteria (AMB), total coliforms (TC), and fecal coliforms (FC) following the methods approved by the FDA's Bacteriological Analytical Manual [30]. All the data obtained in this work was analyzed according to the official 093 guideline [31], which establishes that food samples should only contain up to 5.17 $\log_{10}$ CFU per g (150,000 CFU/g) of AMB and up to 100 MPN/g of FC.

2.3. Isolation and Identification of Mycobacteria. For both RTE foods (salads and sprouts), 45 mL of supernatant from dilution 10^{-1} was placed in sterile conical centrifugation tubes (Falcon type) of 50 mL and then was centrifuged (4,000 ×g at 25°C for 20 min). Supernatants were discarded, and pellets were resuspended in 20 mL of 50 ppm chlorine solution or 20 mL of 0.1% cetylpyridinium chloride, for salads and sprouts, respectively. The suspensions were incubated at room temperature for 30 min and then neutralized with 20 mL of phosphate buffer (pH 7.0). Samples were centrifuged as above and pellets were resuspended in 5 mL of Dubos medium (Difco, Becton Dickinson, Sparks, MD) with albumin-dextrose-catalase (ADC; Becton Dickinson, Mexico); 100 μL of this suspension was inoculated onto Middlebrook 7H10 agar (Difco, Becton Dickinson) supplemented with ADC, cycloheximide (500 μg/mL), and the PANTA cocktail (Becton Dickinson) (40 U/mL polymyxin B, 4 μg/mL amphotericin B, 16 μg/mL nalidixic acid, 4 μg/mL trimethoprim, and 4 μg/mL azlocillin). Plates were incubated at 35°C and were examined daily for the first eight days and thereafter once a week for two months. Once the bacterial growth had been observed on the Middlebrook 7H10 agar, the identification of acid-fast bacilli was carried out by Ziehl-Neelsen stain. Acid-fast bacilli were subcultured on Middlebrook 7H10 agar, labeled by sampling location and with a consecutive number.

Strains belonging to the genus *Mycobacterium* and to the *M. tuberculosis* complex were identified by two PCR assays previously described [32]. Briefly, 3 μL aliquots of bacterial lysates were subjected to amplification, using a standard *Taq* polymerase (Life Technologies, Rockville, MD) in a total volume of 50 μL of PCR mixture; RAC1 and RAC8 and MTB-F and MTB-R primers [32] were used for identification of the *Mycobacterium* genus and of the strains belonging to the *M. tuberculosis* complex, respectively. The amplicon produced by the primer combination of RAC1 and RAC8 contains the last 99 codons of the *mur*A gene, the promoter region of the *rrn*A operon, and 360 nucleotides from the 5′ end of the 16S rRNA gene. As shown by Perez-Martinez et al. [33], the amplicon size varies depending on the mycobacteria species, from 934 to 1300 bp. MTB-F/MTB-R primers amplified a DNA fragment coding for the last five codons of *mur*A gene, the promoter region of the *rrn*A operon, and the 5′ end of the 16S rRNA; a 488 bp fragment characteristic only for *M. tuberculosis* complex members should be amplified. Therefore, by exclusion, mycobacteria strains that did not belong to the *M. tuberculosis* complex were considered to be NTM. These NTM species were identified by three methods: (i) PCR restriction enzyme pattern analysis (PRA) of the 65 kDa heat shock protein gene (*hsp*65), as described by Telenti et al. [34]; (ii) sequencing of the hypervariable region 2 (V2) of the 16S rRNA gene [35]; and (iii) sequencing of the *rpo*B gene [36]. Mycobacterial PRA was performed by PCR amplification of a 439 bp fragment of the *hsp*65 gene by using primers Tb11 and Tb12 [34]. PCR products were digested in two separate reactions with two restriction enzymes, BstEII (New England Biolabs) and HaeIII (Invitrogen). Digested products were then analyzed using the Agilent 2100 bioanalyzer. DNA 1000 LabChips (Agilent) were used according to manufacturer's protocol. PRA results were interpreted with the algorithm described by Telenti et al. [34], which is available on the PRA database [37].

Identification of the mycobacterial species was also carried out by automatized sequence of the hypervariable region 2 (V2) of the 16S rRNA gene and of the *rpo*B gene. The amplification of the 16S rRNA gene was performed using the RAC1 and RAC8 primers [32]. For the amplification of the *rpo*B gene, the Myco-F and Myco-R primers were used to obtain a product of 723 bp [36]. Both products of PCR were sequenced using the RAC8 [32] and Myco-F [36] primers, respectively, and the big dye terminator ready reaction kit

Table 1: Populations and frequencies of aerobic-mesophilic bacteria (AMB), total coliforms (TC), and fecal coliforms (FC) on RTE-salads and RTE-sprouts samples.

Microorganisms group	Minimum	Median	Maximum	Frequency (%)	Number of samples out of the 093 guideline* (%)
RTE-salads from SPM[a]					
AMB	3	4.9	6.6	50 (100)	21 (42)
TC	<3	56	>1100	46 (92)	NA
FC	<3	<3	210	7 (14)	2 (4)
RTE-salads from SVS[a]					
AMB	3	6.1	6.7	50 (100)	38 (76)
TC	3	1100	>1100	50 (100)	NA
FC	<3	1.5	>1100	25 (50)	6 (12)
RTE-sprouts from SPM[a]					
AMB	6.1	7.4	8	50 (100)	NA
TC	6.1	460	>1100	50 (100)	NA
FC	<3	3.3	>1100	29 (58)	NA
RTE-sprouts from SVS[a]					
AMB	6.1	7.3	8.8	50 (100)	NA
TC	26	1100	>1100	50 (100)	NA
FC	<3	23.5	>1100	40 (80)	NA

[a]$n = 50$. Minimum, median, and maximum values are in $\log_{10}$ CFU per g for aerobic-mesophilic bacteria and in most probable number (MPN) per g for total coliforms and fecal coliforms. SPM: supermarkets, SVS: street-vendor stalls, and NA: not applicable (there is no official guideline for this food).
*Guideline that establishes that food samples should contain up to 5.17 $\log_{10}$ CFU per g (150,000 CFU/g) of AMB and up to 100 MPN/g of FC.

(Perkin-Elmer, Inc., Wellesley, MA). The sequences were analyzed by ABI PRISM 310 genetic analyzer system (Perkin-Elmer). Nucleotide sequences were compared to known sequences in the GenBank database by using the Blastn algorithm. Species identifications were based on the 100% similarity cut-off for the 16S rRNA gene and ≥97% for the *rpo*B gene.

The identification of some of the isolated NTM was not possible using the methods described above; therefore, full-length 16S rRNA gene was amplified using the 13B/8FPL universal primers [38]. Purified PCR products were directly sequenced in both forward and reverse directions using the same primers as for PCR. Nucleotide sequences were compared to known sequences in the GenBank database by using the Blastn algorithm.

2.4. Statistical Analyses. Categorical variables (AMB, TC, and FC) were compared using the $(\text{chi})^2$ test for the 2 categories of origin of samples (SPM and SVS). We calculated P value from Fisher test for corresponding data. The values of median of CFU of AMB and MPN of TC and FC were compared using the Kruskal-Wallis test. Two-tailed probability values were calculated. The point-biserial correlation coefficient (r_{pb}) value was calculated to quantify the relationship between the nominal (presence of NTM) and quantitative variables (concentration of AMB, TC, and FC). Similarly, the phi correlation coefficient (r_{phi}) value was calculated to quantify the relationship between all nominal variables (presence of NTM and AMB, TC, and FC). A P value < 0.05 was considered significant. All statistical analysis was run with the Statistical program SPSS for Windows version 21.

3. Results

The RTE-salad and RTE-sprout samples had unsatisfactory microbiological quality (results outside acceptable microbiological limits, see reference [39]). A total of 59% of RTE-salads (21% from SPM and 38% from SVS, $P = 0.001$) did not comply with the 093 guideline (see Section 2) (Table 1). In contrast, FC was detected in 32% of samples; of these, only 8% exceeded the permissible limit of the official guideline. The median concentration of AMB in RTE-salad samples from SPM was significantly lower than the one observed in those from SVS ($P < 0.001$). Similarly, we found equivalent results when the median concentrations of MPN/g of TC and FC were compared ($P < 0.001$).

AMB and TC were also present in 100% of RTE-sprouts samples analyzed (Table 1); FC were present in 69% of samples. The median concentration of FC was significantly lower in RTE-sprouts from SPM (3.3 MPN/g) than the one found in SVS (23.5 MPN/g) ($P = 0.007$). However, the median concentrations of AMB in samples from SPM and SVS were similar ($P = 0.762$). Likewise, we observed the same result when we compared the median concentration values of TC between samples from SPM and from SVS ($P = 0.169$).

One hundred RTE-salads were tested for the presence of mycobacteria. Seven RTE-salads harbored NTM: three were *M. fortuitum*, two were *M. chelonae*, one was

Table 2: Characteristics of positive samples for NTM and species identified.

Foods	Origin	Number of positive samples, type	Ingredients	Number and species of NTM identified
RTE-salads	SVS	3, mixed	Lettuce, carrot, cucumber, Mexican turnip, tomatoes, onion	2, *M. fortuitum* 1, *M. chelonae*
	SVS	1, mango	Lettuce, mango, Mexican turnip, strawberry	1, *M. fortuitum*
	SVS	3, nopal	Cactus, tomatoes onion, coriander	1, *M. mucogenicum* 1, *M. chelonae* 1, *M. sp*
RTE-sprouts	SPM	3, alfalfa	Alfalfa sprouts	2, *M. abscessus* 1, *M. gordonae*
	SPM	1, alfalfa and clover	Alfalfa sprouts Clover sprouts	1, *M. porcinum*
	SPM	1, alfalfa and onion	Alfalfa sprouts onion sprouts	1, *M. porcinum*
	SVS	4, alfalfa	Alfalfa sprouts	1, *M. avium* complex 3, *M. porcinum*
	SVS	1, alfalfa and soybean	Alfalfa sprouts soybean sprouts	1, *M. mucogenicum*
	SVS	1, soybean	Soybean sprouts	1, *M. mucogenicum*
	SVS	1, broccoli	Broccoli sprouts	1, *M. gordonae*

SVS: street-vendor stalls; SPM: supermarket.

M. mucogenicum, and one was *M. sp* (Table 2). All NTM were isolated from RTE-salads collected in SVS ($P < 0.05$). No correlation between the presence of NTM and the presence of AMB ($r_{phi} = 0.154$, $P = 0.234$), TC ($r_{phi} = 0.056$, $P = 1.000$), and FC ($r_{phi} = 0.064$, $P = 0.453$) was found. Likewise, no correlation between the presence of NTM and the number of AMB ($r_{pb} = 0.098$, $P = 0.330$), TC ($r_{pb} = -0.024$, $P = 0.812$), and FC ($r_{pb} = 0.162$, $P = 0.106$) was observed. Of the 100 RTE-sprout samples analyzed, 12 yielded NTM. *M. porcinum* was the most frequently isolated organism (five isolates). We have also recovered two strains of *M. abscessus*, two of *M. gordonae*, two of *M. mucogenicum*, and one strain belonging to the *M. avium* complex (Table 2). Comparison of the number and species of NTM isolated from SPM and SVS showed no significant differences ($P = 0.424$). We also found no correlation between the presence of NTM and the presence of AMB, TC, and FC ($r_{phi} = -0.081$; $P = 0.475$) in RTE-sprouts. Similarly, we did not observe correlation between the presence of NTM and the median concentration of TC ($r_{pb} = 0.046$, $P = 0.653$) and FC ($r_{pb} = 0.099$, $P = 0.326$). Nevertheless, we did observe a significant correlation between the presence of NTM and the median concentration of AMB ($r_{pb} = 0.331$; $P = 0.001$).

4. Discussion

This study shows that AMB were detected in all RTE-salads regardless of source, with limits ranging from 3 to 6.6 $\log_{10}$ CFU/g. AMB counts were found to be higher than those reported for RTE-salads in Johannesburg, South Africa [40]. In contrast, the number of these microorganisms (AMB) was found to be lower than that reported for RTE-salads from Porto, Portugal, and Catalonia, Spain [41, 42]. The frequency of TC on RTE-salads observed here coincides with previous studies [43–45] carried out both in Mexico and in Brazil, countries where proper raw vegetable product handling and sanitation practices need to be promoted and implemented.

FC were identified in 32% of RTE-salad samples; their frequency was substantially lower than that reported for RTE-salads from other developing countries. For instance, frequencies of 90.5% and 89% of FC were reported from Brazil [45] and Costa Rica [46], respectively. In spite of that, Gómez-Aldapa et al. [43] and Castro-Rosas et al. [44] reported frequencies of FC of 95.5% and 99%, respectively, collected from restaurants in our country. We can assume that these higher numbers are the result of the greater number of people that can possibly be involved in the handling of this type of food in these types of places. Furthermore, these different numbers compared to the ones found by our work may be due also to the quality of water used for growing those vegetables, to the different methods of handling them, and to the different sampling techniques used. Unsatisfactory microbiological state of RTE-salads was more frequently observed in salads from SVS than in those from SPM ($P = 0.001$); we suggest that these SVS lack adequate appreciation of basic food safety issues; that is, street vendors keep the salads at room temperature (up to 27°C) unlike supermarkets, where the salads are stored at refrigerated temperatures (4 to 7°C). Street vendors often use stands that are of inefficient construction; running water is not easily accessible and hand and dish washing are performed in the same bucket, sometimes without soap. Wastewater is usually discarded right there in the streets, and garbage is likewise "conveniently" discarded right next to the stands, providing

attraction, food, and harborage for insects and rodents. In many cases, toilets are not available, thus forcing the vendors to eliminate their body wastes also in areas close by and to return to their vending sites without washing their hands. Such conditions and practices are likely to lead to cross contamination of street food. In other cases, vendors buy raw materials from dubious sources, and these materials may be contaminated with foodborne pathogens [47–49].

Regarding the microbiological quality of the sprouts collected in our work, the frequencies and concentrations of AMB found coincide with previous studies from different countries [3, 42, 50]. It has been reported that vegetable seeds could contain <2 $\log_{10}$ CFU/g of BMA. This naturally occurring population of microorganisms can rapidly increase during germination and sprouting because of the favorable conditions for bacterial growth [3]. Consequently, if seeds become contaminated with a pathogen, the sprouting process provides excellent conditions for consequent growth and distribution.

In our study, TC were detected in 100% of sprouts samples, result which coincides with that reported for bean sprouts in Central Mexico, where *Salmonella* and diarrheagenic *Escherichia coli* pathotypes were also identified [51]. On the other hand, FC were detected in the 69% of our RTE-sprout samples; this frequency was found to be lower than that reported for RTE-sprouts from markets in the town of Pachuca, Mexico [51].

Those high frequencies and concentrations of AMB, TC, and FC found in our RTE-sprouts may mean that they are so heavily contaminated that traditional sanitation practices would not be enough to reduce bacterial contamination. It has been suggested that an alternative, which can be used only by big food companies, for reducing the bacterial load in sprouts is the use of ionizing radiation, since it has been reported that a dose of 1.5 and 2 kGy can significantly reduce *E. coli* O157:H7 and *Salmonella* to nondetectable limits in bean and radish sprouts [52]. Therefore, our results conclude, together with some others [43, 44, 51] reported for our country, the highlighted need for implementing stricter hygienic control standards and measures for vegetables and sprouts grown in Mexico.

In this study, we combined conventional and molecular methods and detected and identified NTM in 7 RTE-salads and 12 RTE-sprouts. All NTM identified in this study have also been found in Mexican water samples [53] and in water samples from other countries [21, 54]. Therefore, it is possible that water may be the original source of NTM transmission to vegetables (used in the salads) or sprouts while growing or during harvesting, washing, slicing, soaking, packaging, and preparation. NTM are opportunistic pathogens found in water and soil as normal flora. Therefore, another possibility of the presence of NTM in RTE-salads or RTE-sprouts comes from the soil and the water used for the growing and the irrigation, respectively, of those vegetables.

A significant correlation between the presence of NTM and the median concentration of AMB ($P = 0.001$) was observed. This may be due to the fact that NTM as well as AMB are environmental organisms that have features in common, such as, growing in moderate to warm temperatures (20 and 45°C) and in aerobiosis conditions.

Some of the NTM identified in our study included species that have been frequently associated with human illness in other countries, that is, *M. avium*, *M. fortuitum*, *M. abscessus*, *M. chelonae*, and *M. mucogenium* [55, 56]. In the case of *M. avium,* it has been suggested that the most common portals of entry of this microorganism are the gastrointestinal and the respiratory tracts [57, 58]. In AIDS patients, *M. avium* is acquired predominantly via the gastrointestinal tract, where it is able to invade the intestinal mucosa, to infect and multiply within submucosal macrophages, and to cause bacteremia, leading to the dissemination of the microorganism to the liver, spleen, and bone marrow [58]. Regarding *M. mucogenium,* it has been associated with infection of the gastrointestinal tract in patients with a diagnosis of Crohn's disease [59].

In Mexico City, the prevalence of NTM infections is poorly known and only a few studies have been published. Among these studies, Lopez-Alvarez et al. [60] reported in 2010 that 15% of mycobacterial strains isolated from 67 HIV patients belonged to NTM (10 strains were identified as *M. avium* and 1 strain was identified as *M. intracellulare*). In another recent study, Cortés-Torres et al. in 2013 [61] reported that 37% of 96 patients in a Mexico City Hospital, suffering from various immunodeficiencies, presented several strains of NTM, including *M. avium*, *M. simiae*, *M. gordonae,* and *M. kansasii*. Although no combined conclusion could be reached between these findings and ours, further studies of DNA fingerprinting of NTM should be carried out, in order to confirm that NTM isolated from RTE-salads and RTE-sprouts are the same as those isolated from patients.

5. Conclusions

Most RTE-salads and RTE-sprouts analyzed in this study had unsatisfactory microbiological quality and some harbored NTM associated with illness. Measures to diminish or eliminate NTM strains from these food items might be advisable, such as a proper handling and washing before consumption of these products. RTE-salads and sprouts could be considered as potential sources for NTM infections in humans.

Conflict of Interests

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

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and EDI, IPN, and Elizabeth Fernandez-Rendon is fellow of COFAA and EDD, IPN.

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Research Article

Antibiotic-Resistant Vibrios in Farmed Shrimp

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Antimicrobial susceptibility pattern was determined in 100 strains of *Vibrio* isolated from the *Litopenaeus vannamei* shrimp and identified phenotypically. A high antibiotic-resistance index (75%) was observed, with the following phenotypic profiles: monoresistance ($n = 42$), cross-resistance to β-lactams ($n = 20$) and multiple resistance ($n = 13$). Plasmid resistance was characterized for penicillin ($n = 11$), penicillin + ampicillin ($n = 1$), penicillin + aztreonam ($n = 1$), and ampicillin ($n = 1$). Resistance to antimicrobial drugs by the other strains ($n = 86$) was possibly mediated by chromosomal genes. The findings of this study support the conclusion that the cultured shrimps can be vehicles of vibrios resistant to β-lactam and tetracycline.

1. Introduction

Bacteria of the *Vibrio* genus occur naturally in marine, estuarine, and freshwater environments [1] and are notably described as shrimp pathogens [2]. Studies indicate that the antibiotic resistance of vibrios isolated from penaeid culture environment is not unusual [3–5], a fact that apparently constitutes a problem to this type of aquaculture activity [6]. In addition, there is the risk of environmental impact, since the use of antibacterial agents as prophylactic measure in aquaculture favors the selection of resistant bacteria, increasing the probability of transferring resistant genes to human pathogens and land animals [7].

Considering the importance of researching the occurrence of antibiotic-resistant bacteria in marine invertebrates intended for human consumption, this study aimed to determine the susceptibility pattern to antibacterial drugs of vibrios isolated from the hemolymph of *Litopenaeus vannamei* shrimp.

2. Materials and Methods

A total of 100 *Vibrio* strains from the bacterial collection of the Microbiology Laboratory at the Environmental and Fish-Sea Sciences Institute (LABOMAR-UFC) were used in this experiment. Only strains previously identified by phenotyping were used [8]. The 100 strains, isolated from the hemolymph of *Litopenaeus vannamei*, were subjected to identification by a set of biochemical keys [9], using the following tests: arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, oxidase, indole, *ortho*-nitrophenyl-β-galactoside (ONPG), Voges-Proskauer, D-glucosamine cs, growth at 0%, 3%, 8%, and 10% NaCl, growth at 40° and 4°C, citrate, gelatinase, urease, resistance to O/129, and acid from glucose, sucrose, arabinose, mannitol, and melibiose. The strains had consonant phenotypic profiles with the species *V. navarrensis* ($n = 53$), *V. brasiliensis* ($n = 15$), *V. parahaemolyticus* ($n = 10$), *V. xuii* ($n = 8$), *V. coralliilyticus* ($n = 5$), *V. cholerae* ($n = 4$), *V. neptunius* ($n = 2$), *V. alginolyticus* ($n = 1$), *V. diazotrophicus* ($n = 1$), and *V. vulnificus* B3 ($n = 1$).

All isolates ($n = 100$) were submitted to antibiotic susceptibility pattern tests by disk diffusion method [9]. For the present study, antibiotics used in shrimp industry [4] and human clinical [10] were selected. The following antimicrobials (Laborclin) were tested: nalidixic acid (Nal 30 μg), ampicillin (Amp 10 μg), aztreonam (Atm 30 μg), cephalothin (Cpl 30 μg), ceftriaxone (Cro 30 μg), ciprofloxacin (Cip 5 μg), chloramphenicol (Clo 30 μg), streptomycin (Est 10 μg),

Table 1: Antimicrobial resistance in vibrios isolated from the hemolymph of *Litopenaeus vannamei* shrimp.

Species	*n*	*n*R (%)	Resistance					
			Pen	Tcy	Cpl	Amp	Atm	Cro
V. navarrensis	53	38 (71,7)	32	—	3	2	7	1
V. brasiliensis	15	9 (60)	9	—	4	1	—	—
V. parahaemolyticus	10	10 (100)	10	9	1	3	—	—
V. xuii	8	8 (100)	8	—	4	2	—	—
V. coralliilyticus	5	3 (60)	3	—	—	—	—	—
V. cholerae	4	4 (100)	4	3	—	2	1	—
V. neptunius	2	0 (0)	—	—	—	—	—	—
V. alginolyticus	1	1 (100)	1	—	—	—	—	—
V. diazotrophicus	1	1 (100)	—	—	—	—	1	—
V. vulnificus B3	1	1 (100)	1	1	—	—	—	
Total	**100**	**75**	**68**	**12**	**12**	**10**	**9**	**1**

*n: number of isolates. nR: number of resistant isolates. Pen: penicillin G; Tcy: tetracycline; Amp: ampicillin; Cpl: cephalothin; Atm: aztreonam; Cro: ceftriaxone.

gentamicin (Gen 10 μg), imipenem (Ipm 10 μg), nitrofurantoin (Nit 300 μg), penicillin (Pen 10 U) sulfazotrim (Sut 25 μg), and tetracycline (Tcy 30 μg). For the antibiogram test, bacterial density was previously adjusted to a 10^8 UFC mL^{-1} concentration, by bacterial suspension in saline solution with 1% turbidity equivalent to the McFarland nephelometer scale 0.5. Suspensions with standard densities were inoculated with "swab" in Petri dishes containing Mueller-Hinton Agar (Difco) medium with 1% NaCl, and then antibiotic disks (Laborclin) were applied. The plates were incubated at 35°C/24 h. The inhibition halos were measured (mm) with digital caliper (Digmess) and since there are no breakpoints defined for *Vibrio*, we use the zone diameter interpretive standards for Enterobacteriaceae cited by CLSI [11].

In order to determine the antibiotic resistance mediation, resistant strains were selected and subjected to plasmid curing by acridine orange (Sigma A-6014) [12]. All resistant strains were grown in Luria Bertani broth, supplemented with 0.100 mg mL^{-1} of acridine orange, incubated at 35°C for 24 h. Subsequently, inocula were removed and pour-plated in TSA containing 1% NaCl, incubated at 35°C for 24 h. From TSA growth, antibiogram was performed as mentioned above. The resistance was considered chromosomal when observed after the curing procedure; otherwise, it was characterized as plasmid.

3. Results and Discussion

The strains used in this study were subjected to biochemical identification only. For the genus *Vibrio*, the phenotypic identification is still considered not enough, since many species can be misidentified. However, the identification of all strains used was validated [9] and previously published [8].

The resistance to at least one antibiotic was observed in 75 strains (Table 1). The only species susceptible to all drugs was *V. neptunius*. In contrast, resistance was verified and confirmed in all *V. parahaemolyticus*, *V. xuii*, *V. cholerae*, *V. alginolyticus*, *V. diazotrophicus*, and *V. vulnificus* B3 strains. The isolates of *V. navarrensis*, *V. brasiliensis*, and *V. coralliilyticus* showed high resistance of 71.7%, 60%, and 60%, respectively.

Table 2 describes data related to nine profiles of 75 resistant vibrios. The most frequent profile was the resistance to penicillin alone (n = 42), followed by the cross-resistance to β-lactams (n = 20) and the multidrug resistance (n = 13).

Vibrios antibiotic-resistant have been detected in different species of shrimp [4, 6, 13–16]. The expression of this type of resistance is often related to inappropriate use of antibacterial drugs in aquaculture [17, 18].

In a research on antimicrobial susceptibility pattern of *V. parahaemolyticus* derived from shrimp, Bhattacharya et al. [3] revealed the occurrence of strains resistant to ampicillin and sensitive to nalidixic acid and nitrofurantoin. These findings may be compared to the ones from this study, since three strains of *V. parahaemolyticus* were resistant to ampicillin, and all were sensitive to the other antibiotics tested (Table 1). Expression of ampicillin [19] and tetracycline [20] resistance by vibrios from shrimp farming regions has been reported.

Recently, Laganà et al. [21] isolated from Italian aquaculture (fish, shellfish, and crustaceans) sites bacteria (*Vibrio* spp. and *Photobacterium damsela* spp. *piscicida*) resistant to β-lactams (ampicillin, carbenicillin, mezlocillin, piperacillin, cephalothin, cefazolin, cefuroxime, cefoxitin, ceftazidime, and aztreonam), quinolones (cinoxacin, nalidixic acid, oxolinic acid, and pipemidic acid), potentiated sulfonamides (sulfamethoxazole + trimethoprim), polymyxin (colistin sulphate), fosfomycin, tetracycline, and RNA synthesis inhibitors (rifampicin). Our results also showed levels of resistance to beta-lactam antibiotics, including cross-resistance (Pen + Cpl, Pen + Atm, Pen + Amp, Cro + Atm, and Pen + Cpl + Amp) (Table 2).

In Brazil, *Vibrio* resistant to antibacterial drugs has been detected in farmed shrimp and cultivation area. Costa et al. [22] detected *Vibrio* strains resistant to ampicillin, sulfazotrim, and ceftriaxone in samples of *Litopenaeus vannamei* shrimp and suggested that the penaeid and its culture environment may constitute the main sources of resistant bacteria. In the present study, a strain with cross-resistance to ceftriaxone and aztreonam was verified; however, there was no resistance to sulfazotrim. Melo et al. [23] reported rates of 90% and 60% antibiotic resistance to ampicillin and amikacin in *V. parahaemolyticus* strains isolated from pond-reared *L. vannamei*. Helena Rebouças et al. [10] showed a high incidence of resistance to ampicillin (45.2%) and to the tetracycline class (38.7%) in vibrios isolated from marine shrimp farming environments.

The high rate (68%) of resistance to penicillin G observed in the present study should be emphasized (Tables 1 and 2). The occurrence of penicillin-resistant vibrios has already been reported in different penaeid culture areas and regions [6, 24]. Srinivasan and Ramasamy [25] detected 100% resistance to penicillin G in vibrios species associated with viral diseased shrimp in India and alert to the environmental, economic, and management problems that may result from the emergence of drug resistant microbial diseases in aquaculture.

Table 2: Antimicrobial resistance pattern in vibrios isolated from the hemolymph of *Litopenaeus vannamei* shrimp.

Classification	Profile	n	Species (number of resistant isolates)
Monoresistance ($n = 42$)	Pen	38	*V. navarrensis* (28), *V. xuii* (4), *V. coralliilyticus* (3), *V. brasiliensis* (2), and *V. alginolyticus* (1)
	Atm	4	*V. navarrensis* (3) and *V. diazotrophicus* (1)
Cross-resistance to β-lactam ($n = 20$)	Pen + Cpl	9	*V. navarrensis* (3), *V. brasiliensis* (3), *V. xuii* (2), and *V. parahaemolyticus* (1)
	Pen + Atm	5	*V. navarrensis* (4) and *V. cholerae* (1)
	Pen + Amp	2	*V. navarrensis* (2)
	Cro + Atm	1	*V. navarrensis* (1)
	Pen + Cpl + Amp	3	*V. xuii* (2) and *V. brasiliensis* (1)
Multiple resistance ($n = 13$)	Pen + Tcy	8	*V. parahaemolyticus* (6), *V. cholerae* (1), and *V. vulnificus* (1)
	Pen + Tcy + Amp	5	*V. parahaemolyticus* (3) and *V. cholerae* (2)

*n: number of resistant isolates; Pen: penicillin G; Tcy: tetracycline; Amp: ampicillin; Cpl: cephalothin; Atm: aztreonam; Cro: ceftriaxone.

Table 3: Plasmid mediation of antimicrobial resistance in vibrios isolated from the hemolymph of *Litopenaeus vannamei* shrimp.

Strain code	Species	Resistance profile	Posthealing profile	
			Resistant	Sensitive
#1	*V. coralliilyticus*	Pen	—	Pen
#2	*V. navarrensis*	Pen	—	Pen
#3	*V. navarrensis*	Pen + Amp	—	Pen + Amp
#4	*V. navarrensis*	Pen + Amp	Pen	Amp
#9	*V. navarrensis*	Pen	—	Pen
#10	*V. navarrensis*	Pen	—	Pen
#17	*V. navarrensis*	Pen + Atm	—	Pen + Atm
#18	*V. navarrensis*	Pen + Atm	Atm	Pen
#36	*V. navarrensis*	Pen	—	Pen
#56	*V. navarrensis*	Pen	—	Pen
#69	*V. navarrensis*	Pen + Atm	Atm	Pen
#74	*V. brasiliensis*	Pen	—	Pen
#94	*V. brasiliensis*	Pen	—	Pen
#98	*V. brasiliensis*	Pen	—	Pen

*Pen: penicillin; Amp: ampicillin; Atm: aztreonam.

Plasmid resistance was characterized for Pen ($n = 11$), Pen + Amp ($n = 1$), Pen + Atm ($n = 1$), and Amp ($n = 1$) (Table 3). Resistance to antimicrobial drugs by the other strains ($n = 86$) was possibly mediated by chromosomal genes.

In the present study only phenotypic detection of plasmids was performed. The curing by acridines has been used since 1960s and normally involves loss of the whole plasmid [26]. In 1970s, Dastidar et al. [27] demonstrated the efficiency of acridine orange in the elimination of R plasmids in *Vibrio cholerae* multidrug-resistant strains. The acridine orange eliminates plasmids from prokaryotic cells [28], and although the conventional methods for curing plasmids by curing agents may induce mutations in the host chromosomal DNA [29], its use in plasmid detection is still being reported in clinical strains [30] and animal/environmental isolates [11, 31, 32].

In 13 strains, the resistance to penicillin G was characterized as plasmid (Table 3). In bacteria of the genus *Vibrio*, the existence of penicillin-resistant encoding plasmid was suggested by Reid and Amyes [33], who described plasmid SAR-1 as capable of hydrolyzing the antibiotics carbenicillin and penicillin G. According to the authors, the most common resistance mechanism to β-lactam antibiotics is the production of β-lactamase enzymes, which hydrolyze the antibiotic and inactivate it.

We detected two strains with the plasmid resistance to ampicillin (Table 3). Teo et al. [34] associated the ampicillin resistance in vibrios with a possible via of mediation by β-lactamase *bla*VHW-1 and *bla*VHH-1 genes of approximately 60 kb present in plasmids.

Molina-Aja et al. [12], in a study of plasmid pattern and antibiotic resistance in *Vibrio* strains isolated from penaeid, detected an incidence of isolates resistant to cephalothin higher than the present study, with 36.1% of the strains carrying resistance to this β-lactam. The same authors attributed to a 21,226 pb plasmid the ability to encode the resistance to cephalothin, thus characterizing that resistance as plasmid. This result should not be compared to the ones in the present study, since the resistance to cephalothin observed in 12 strains was, possibly, of chromosome nature.

4. Conclusions

The findings of this study support the conclusion that the cultured shrimps can be vehicles of vibrios resistant to β-lactam and tetracycline. Thus, the emergence of resistant bacteria to antibacterial drugs may be indicative of the indiscriminate use of these drugs in the cultivation of aquatic organisms. Furthermore, the detection of resistance mediated by mobile genetic elements, plasmids, serves as alert to the possibility of horizontal transfer of antibiotic resistance genes among bacteria.

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

Listeriosis Outbreaks in British Columbia, Canada, Caused by Soft Ripened Cheese Contaminated from Environmental Sources

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Soft ripened cheese (SRC) caused over 130 foodborne illnesses in British Columbia (BC), Canada, during two separate listeriosis outbreaks. Multiple agencies investigated the events that lead to cheese contamination with *Listeria monocytogenes (L.m.)*, an environmentally ubiquitous foodborne pathogen. In both outbreaks pasteurized milk and the pasteurization process were ruled out as sources of contamination. In outbreak A, environmental transmission of *L.m.* likely occurred from farm animals to personnel to culture solutions used during cheese production. In outbreak B, birds were identified as likely contaminating the dairy plant's water supply and cheese during the curd-washing step. Issues noted during outbreak A included the risks of operating a dairy plant in a farm environment, potential for transfer of *L.m.* from the farm environment to the plant via shared toilet facilities, failure to clean and sanitize culture spray bottles, and cross-contamination during cheese aging. *L.m.* contamination in outbreak B was traced to wild swallows defecating in the plant's open cistern water reservoir and a multibarrier failure in the water disinfection system. These outbreaks led to enhanced inspection and surveillance of cheese plants, test and release programs for all SRC manufactured in BC, improvements in plant design and prevention programs, and reduced listeriosis incidence.

1. Introduction

Listeria is an environmentally ubiquitous Gram positive bacterium found in soil and vegetation, sewage, water, animal feeds, and food processing environments [1]. The pathogenic species *L. monocytogenes* (*L.m.*) infects domestic animals (i.e., cattle, sheep, goats, horses, poultry) and has also been found in wild avians, fish, and shellfish [2]. Of the eight species, *L.m.* is most often associated with human illness, although *L. ivanovii*, common in ruminant infections, is occasionally associated with human infection as well [3]. In humans, 99% of listeriosis cases are contracted through the consumption of contaminated food [4]. Healthy individuals rarely seek medical care for listeriosis infections, as these are self-limited with gastroenteritis and/or mild flu-like symptoms. However, elderly persons (>65 years), immune compromised individuals, neonates, and pregnant women and their fetuses are more susceptible to invasive forms of listeriosis infections, which can lead to encephalitis, meningitis, septicemia, and/or spontaneous abortions during the last trimester of pregnancy. Mortality rates for invasive listeriosis typically range between 20% and 40% [5, 6].

Several categories of ready-to-eat (RTE) foods have been associated with listeriosis outbreaks including vegetables (corn, celery, coleslaw, sprouts/taco salad) [6–9]; fruits (cantaloupe) [10]; processed deli meats (beef, turkey, hog head cheese, hot-dogs, cooked ham, jellied pork, RTE sandwiches) [11–17]; seafoods (crab meat, cold-smoked trout, smoked mussels, shrimp) [6, 18–20]; unpasteurized dairy products (Mexican soft cheese, raw milk cheeses, on farm fresh cheese) [21–25]; pasteurized dairy products (butter, soft cheese, sour milk curd cheese, fluid milk) [26–32].

Control of *L.m.* in food processing and retail environments is particularly difficult, due to its high cold tolerance

(i.e., growth at refrigeration temperatures as low as −1.5°C) and its ability to form environmentally stable biofilms resistant to sanitation [6, 33–35]. In particular, in dairy milk and cheese processing, *L.m.* contamination may occur during transfer of raw fluid milk into the processing facility, from inadequate pasteurization, and from postpasteurization contamination during one or more of the following steps: addition of culture, cheese, curd formation, cutting, stirring, washing, moulding, draining, pressing, brining, salting, ripening, and packaging [36]. It has also been demonstrated that pathogens can be transferred from dairy animals to dairy processing plants [23], although cross-contamination of milking areas can be minimized [37]. *L.m.* contamination may also be introduced via poor employee hygiene, via poor plant design, via equipment malfunction, from other nondairy ingredients (spices, starter cultures, water), and from inadequate sanitation and pest control. Proximity to farm environments may also be a risk factor for the introduction of *L.m.* into dairy processing plants, as increased incidence of *L.m.* has been linked to dairy farms with poor hygienic practices [38].

In the province of British Columbia (BC), Canada, cheese manufacture is regulated under the Milk Industry Act and Milk Industry Standard Regulations [39]. In BC, soft ripened cheese (SRC) aged for less than 60 days is only permitted to be made from pasteurized milk, reducing the risk of pathogen contamination through raw milk, although one other province (Quebec) allows the manufacture of raw milk soft ripened cheese. Two programs are used to control *L.m.* and other hazards in BC dairy plants: prerequisite programs and a Hazard Analysis Critical Control Point (HACCP) program [40, 41]. An effective HACCP program includes monitoring and control of critical control points established for the dairy pasteurization and postpasteurization process steps listed above. Effective prerequisite programs, also known as good manufacturing practices (GMPs), control the other sources of contamination found within and outside the dairy processing plant, such as employee hygiene and sanitation. Inadequate identification of food safety hazards, poor control of the processing environment, and lapses in carrying out established procedures can lead to food safety failures, allowing contamination to occur during cheese manufacturing steps. Both HACCP and prerequisite programs/GMPs must be functional for the safe manufacture of cheese.

Unlike harder types of cheese, SRC can be more vulnerable to postpasteurization bacterial contamination and subsequent outgrowth due to low acidity and high moisture content. For example, a Camembert would have 70% moisture and pH range of 5.5 to 5.8, whereas a harder Cheddar cheese would have 42% moisture and pH of 5.45 [42, 43]. The motile *L.m.* readily grows and multiplies in the cheese substrate and can penetrate SRCs [44]. During the ripening stage, SRC is typically held at 10°C and undergoes increasing alkalinity caused by the growth of bacteria and moulds on the surface of the cheese rind [42]. After ripening, SRC is refrigerated up to three months creating favourable conditions for the growth of cold-tolerant *L.m.* [44].

Although in theory many critical control points and potential failures in dairy processing have been described during the production of SRC, very few outbreak investigations have successfully identified and described the route of contamination of SRC during postpasteurization processing steps. Previous outbreaks involving pasteurized SRC have shown issues with cross-contamination at retail and by food handlers [28, 45, 46]. This paper describes the investigation of two separate pasteurized SRC outbreaks that occurred in BC, Canada, in 2002 that led to the uncovering of two novel environmental transmission pathways affecting postpasteurization processing.

2. Materials and Methods

Two listeriosis outbreaks (A and B) occurred in 2002 in BC, one in February 2002 (Plant A) and one in September 2002 (Plant B). Each outbreak was investigated by three means: via epidemiologic methods, laboratory analyses of samples, and plant investigations. Public health responses, in the form of health advisories and recalls, occurred as required by the investigation findings.

2.1. Epidemiologic Investigations. A multiagency investigation was required for both outbreaks. The communicable disease department of the BC Centre for Disease Control (BCCDC) coordinated case finding and followup, as well as the necessary restaurant and retail inspections which involved multiple health authorities. In addition to passive identification of cases self-reporting illness to their physician or emergency department, active case identification occurred following release of public health advisories that described the cheese implicated in the outbreaks. The information collected from cases included case demographics (age and gender), symptoms and illness onset, a description of exposure history, and other possible food vehicles. Cases were defined as symptomatic individuals exposed to the implicated SRC in their food history within the exposure period (up to 70 days after ingestion of the SRC). Confirmed invasive cases were identified through isolation of *L.m.* from a normally sterile site (i.e., blood or cerebral spinal fluid (CSF)) and had a compatible *L.m.* serotype and pulsed field gel electrophoresis (PFGE) biotype to the SRC; clinical cases presented with febrile gastroenteritis (vomiting, diarrhoea, and abdominal cramps) with or without positive stool identification of *L.m.* Confirmed noninvasive clinical cases with *L.m.* positive stool had a compatible *L.m.* serotype and PFGE biotype to the SRC.

2.2. Laboratory Investigations. Clinical (i.e., stool, blood, CSF), food (i.e., cheese and ingredients), water, and environmental samples (i.e., swabs from the plant, soil samples, animal faecal samples, compost, animal bedding, swabs from the farm environment and others) were tested according to standard culture methods. Briefly, any bacterial smears from sterile sites of suspected cases were referred to the provincial diagnostic laboratory for confirmation if they showed growth of *L.m.* on blood agar plates. Stool samples were screened for a variety of bacterial pathogens on respective selective enrichment agars: *L.m.*, other Gram positive pathogens (*Bacillus*, *Staphylococcus*), and routine enteric gram negative pathogens (*Salmonella*, *Shigella*, *Escherichia coli*, *Yersinia*, *Aeromonas*,

and *Campylobacter*). In addition, screening for norovirus was conducted using reverse-transcription polymerase chain reaction [47]. Swabs of stool were cultured for *Listeria* via cold enrichment in *Listeria* enrichment broth for a minimum of 24 h followed by streaking bacteria onto selective agars of PALCAM, Oxford, and LPM (lithium chloride phenylethanol moxalactam) and incubation at 30°C for up to 48 h. Food, water, and environmental samples were assayed according to Health Canada's established procedures for *Listeria* in foods and environmental samples (MFHPB-30) and others [48–52]. Positive isolates were further characterized by serotyping and PFGE by the BCCDC Public Health Microbiology & Reference Laboratory and the National Microbiology Laboratory, as described elsewhere [53, 54]. In addition to *Listeria* testing, standard plate counts (SPC) and phosphatase tests were performed on raw milk samples; water samples were tested for heterotrophic plate counts (HPC) and total coliforms (TC). Additional testing of water colour and ultraviolet (UV) light absorbance and transmittance was conducted during the investigation of plant B [55, 56].

2.3. Plant Investigations. On-site inspections of each implicated dairy processing plant were conducted. Types and quantities of cheese manufactured at the plants from various milk sources were assessed for contamination, processing failure risks, and adherence to HACCP, prerequisite, programs and GMPs. Inspection focused on (1) raw milk quality and handling, (2) pasteurization effectiveness, (3) pasteurization procedures, (4) pasteurization equipment, and the likelihood of postpasteurization contamination from (5) raw milk, (6) the plant environment, (7) ingredients, and (8) personnel. Inspections included owner and operator interviews, review of onsite records and procedures, direct observation of processes involved during SRC production, testing of the dairy environment, testing of dairy ingredients (including water), and finished product sampling at the dairy processing plants. Further site inspections were conducted based on preliminary laboratory findings, and based on hypotheses that arose during the investigation.

3. Results

3.1. Epidemiologic Results. In the first of the two outbreaks, two initial cases of bacterial meningitis caused by *L.m.* with onset of illness on February 3 and 7, 2002, respectively, provided a history of consumption of cheese produced by plant A and sold in a large farmers' market in Vancouver held on February 1–3, 2002. A Canadian Food Inspection Agency recall of all cheese manufactured in plant A was initiated on February 13, 2002. Trace-back activities revealed that 14 cheese varieties from plant A were sold to 20 different restaurant and retail premises on Vancouver Island and in Vancouver in the weeks prior to the outbreak (outbreak A). A total of 48 illnesses were linked to this outbreak: 43 cases with febrile gastroenteritis, three meningitis cases, and two cases of bacteremia in pregnancy (Table 1, Figure 1). The majority of illnesses were in females (64%), with ages ranging from four to 85 years (median 49 years). The median incubation period

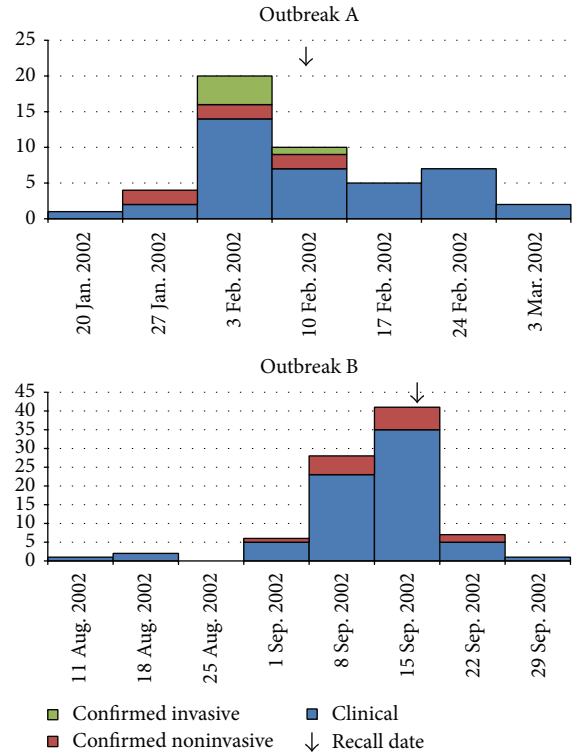

Figure 1: Epidemiological curves of weekly illness onsets for confirmed and clinical listeriosis in 2002 outbreaks.

was seven days (range 1–33 days). The *L.m.* strain detected in food and clinical samples in this outbreak was serotyped as 4b, with PFGE pattern LMAAI.0140 (*Apa* I) and LMACI.0023 (*Asc* I). This *L.m.* strain was detected in five invasive (sterile site sample) and six noninvasive (stool sample) cases.

In the second outbreak, cheeses produced by plant B were sold at a farmers' market on Vancouver Island on September 6, 2002, and linked to a cluster of five illnesses within a family with rapid onset (<24 hrs) of severe febrile diarrhoea requiring hospitalization. An investigation began on September 18, one day following notification and receipt of food and clinical samples, and implicated cheese was recalled on September 19. A total of 86 cases, all with febrile gastroenteritis, were linked to this outbreak, with the earliest case identified one month prior, on August 15, 2002 (Table 1, Figure 1). The majority of illnesses were in females (72%), with ages ranging from 14 to 76 years (median 46 years). The median incubation period was two days from product consumption to onset of symptoms. The *L.m.* strain detected in food and clinical samples in this outbreak was serotyped as 4b, with PFGE pattern LMAAI.0017 (*Apa* I) and LMACI.0082 (*Asc* I). This *L.m.* strain was detected in 14 noninvasive (stool sample) cases.

3.2. Food and Environmental Testing Results. Overall, 113 and 104 food and environmental samples were collected and tested for the presence of *Listeria* spp. during outbreak A and B investigations, respectively. Additional sampling of raw milk in both outbreaks did not detect *L.m.* in raw milk sources and bacterial SPC were below the provincial standard of

Table 1: Summary of outbreak findings.

	Outbreak A	Outbreak B
Illnesses		
Total number of illnesses	49	86
First reported illness	February 3, 2002	August 15, 2002
Organism identified	*L.m.*	*L.m.*
Serotype	4b	4b
PFGE designations—Apa I	LMAAI.0140	LMAAI.0017
—Asc I	LMACI.0023	LMACI.0082
Number of noninvasive (clinical) cases		
Febrile gastroenteritis (stool+)	44 (6)	86 (14)
Number of invasive (confirmed) cases		
Meningitis	3	0
Bacteremia in pregnancy	2	0
Demographics		
Age range in years (median)	4 to 85 (49)	14 to 76 (46)
% Female	64	72
Clinical findings		
Incubation period in days (median)	1 to 33 (7)	0.5 to 28 (2)
Symptoms reported (%)		
Fatigue	51	62
Myalgia	46	55
Chills	38	0
Night sweats	24	0
Bone pain	19	0
Abdominal pain	8	54
Cheese[1] prepared at plant		
Number of cheese types produced	14	10+
Bacterial smear surface soft ripened cheese	**Yes**[2*]	Yes
Chevre (goat milk soft cheese)	**Yes**[*]	No
Curds (e.g., cheddar)	**Yes**[*]	Yes
Feta cheese	Yes	Yes
Soft cheese (e.g., fromage frais)	Yes	Yes
Hard cheese (e.g., cheddar cheese)	**Yes**[*]	Yes
Semihard cheese (e.g., raclette)	No	**Yes**[*]
Soft mould ripened cheese	**Yes**[*]	**Yes**[*]
Investigation findings (Acceptable/neutral/unacceptable)		
Raw milk quality and handling	Acceptable	Acceptable
Pasteurization effectiveness/procedures	Acceptable	Acceptable
Pasteurization equipment	Acceptable	Acceptable
Postpasteurization—raw milk contamination	**Neutral**	Acceptable
Postpasteurization—interior plant environment	Acceptable	Acceptable
Postpasteurization—ingredients	**Unacceptable**	**Unacceptable**
Postpasteurization—personnel	**Unacceptable**	Acceptable
External environment	**Neutral**	**Unacceptable**

[1]Cheese types made with cow *or* goat milk unless specified; [2]cheese products linked to illness are indicated in bold with[*]; *L.m.*, *Listeria monocytogenes*.

4.70 $\log_{10}$ CFU/mL [57]. Phosphatase tests on pasteurized milk and cheese products were also negative in both outbreaks (Table 2).

3.2.1. Plant A Results. L.m. positive cheese types associated with illnesses in outbreak A included those made of cow's and goat's milk, specifically SRC, fresh curds, a hard cheddar cheese, and chevre. Within the positive SRC, two varieties of cow cheese (tommes—a bacterial-smear ripened soft cheese and camembert—a soft-mould ripened cheese) and two varieties of goat cheese (same types) with one or more brand name(s) for each variety were distributed to restaurant and

Table 2: Results of lab tests in milk, cheese, and environmental samples.

	Outbreak A	Outbreak B
Milk samples		
Raw milk SPC ($\log_{10}$ CFU/mL)		
(1) Government dairy pool	3.90	n/a
(2) Local farm—cow	n/a	3.00
(3) Local farm—goat	4.43	n/a
Raw cow milk *L.m.*	Absent	Absent
Raw cow milk pH	NT	6.8
Pasteurized cow milk phosphatase	Negative	Negative
Cheese samples		
Number of cheese samples *L.m.* + (tested)	16 (25)	22 (29)
Number of varieties + (number of lots+)	8 (12)	3 (3)
L.m. counts (median $\log_{10}$ CFU/g)	2.0	2.0
L.m. counts (range $\log_{10}$ CFU/g)	<2 to 9.4	<2 to 9.0
Environmental samples		
In the plant—ingredients		
Number of *L.* spp. + (tested)	2 (33)	2 (32)
In the plant—surfaces		
FCS number of *L* spp. + (tested)	3 (17)	0 (5)
NFCS number of *L* spp. + (tested)	2 (23)	0 (17)
Outside the plant		
Number of *L* spp. + (tested)	1 (1)	4 (7)
On the hobby or dairy farm		
Number of *L* spp. + (tested)	12 (14)	6 (14)
Number of environmental *L.m.* + (tested)	17 (88)	14 (75)

SPC, standard plate count; NT, not tested; n/a, not applicable; *L.m.*, *Listeria monocytogenes*; *L.* spp., *Listeria* species (*L. innocua, L. ivanovii* or *L. seeligeri*); FCS, food contact surface; NFCS, nonfood contact surface.

retail premises. A cheddar cheese, chevre made with goat's milk, and fresh cheese curds made with cow's milk were associated with illness. Several varieties were made and/or packaged on different dates, with between 8 and 12 distinct lot codes, spanning several weeks. The majority of cheese recovered (64%, $n = 25$) tested positive for *L.m.*, and all samples matched the outbreak serotype and PFGE pattern. Bacterial counts of *L.m.* ranged from <2 to 9.4 $\log_{10}$ CFU/g (median counts 2.0 $\log_{10}$ CFU/g) (Table 2).

Overall, *L.m.* was detected in 17 (19.3%) of the 88 environmental samples collected and included samples of ingredients, plant surfaces (e.g., food contact surfaces where cheese was aged and nonfood contact surfaces, such as drains, air vents), and the grounds area around plant A, including the adjacent hobby farm. Within the plant, seven samples were *L.m.* positive (9.6%, $n = 73$), all from a cheese aging room. These samples included two culture solutions stored in spray bottles, the shelf for storage of the spray bottles, condensate from the aging room blower unit, and a plastic bin where cheese was aged. *L.m.* counts of 7.3 $\log_{10}$ CFU/mL and 2.9 $\log_{10}$ CFU/mL were detected in *Penicillium* and *Brevibacterium* spray culture solutions, respectively. Outside the plant, 13 samples collected were found positive for *L.m.* (87%, $n = 15$), including a grass walkway leading to the plant, pig, and chicken areas on the farm. Other *Listeria* spp. (*L. innocua*) were also detected in these areas (Table 3).

3.2.2. Plant B Results. *L.m.* positive cheese types associated with illnesses in outbreak B were limited to two varieties: a semihard cheese (raclette) and two varieties of SRC. Three lot/date codes were implicated. Contamination rates in these types of cheese were also high (76%, $n = 29$), with counts of *L.m.* ranging from <2 to 9.0 $\log_{10}$ CFU/g (median counts 2.0 $\log_{10}$ CFU/g), all of which matched the outbreak serotype and PFGE pattern (Table 2). Single lots of two cheese varieties were likely contaminated by a single lot of one SRC variety. These *L.m.* positive types of cheese were packaged with the single lot of contaminated SRC side by side on a cheese cutting board for sale at retail. *L.m.* counts in these types of cheese ranged from <2 $\log_{10}$ to 4.78 $\log_{10}$ CFU per gram, demonstrating the ability of the surface *L.m.* in the contaminated SRC to transfer to other types of cheese and multiply rapidly.

Overall, *L.m.* was detected in 14 (18.7%) of the 75 environmental samples collected and included one hydrated ingredient sample, water samples in and outside of the plant, and a variety of farm samples. Within the plant, *Listeria* spp. (*L. seeligeri*) were detected in two (6.2%) of the ingredients used in cheese production in plant B, a water sample taken from inside the plant (post-UV water treatment), and a hydrated mould culture prepared on September 16. All other plant surfaces ($n = 22$) and ingredients ($n = 30$) were negative for *Listeria* spp. Outside the plant and on the dairy farm, several different species of *Listeria* were detected, including *L.m.*,

Table 3: *Listeria* spp. detailed results from investigations.

Sample description	*Listeria* spp.	Matched to cheese *L.m.* strain?
Outbreak A samples		
Brevibacterium culture spray solution	*L. monocytogenes*	Yes
Penicillium mould spray solution	*L. monocytogenes*	Yes
Aging room shelf (where spray bottles stored)	*L. monocytogenes*	NT[1]
Aging room—inside plastic aging containers (3 samples)	*L. monocytogenes*	Yes
Aging room—condensate from blower unit	*L. monocytogenes*	Yes
Whey trench outside	*L. monocytogenes*	NT
Grass beside walkway	*L. monocytogenes*	NT
Pig garden—poo area	*L. monocytogenes*	NT
Pig garden—wet bedding	*L. innocua*	
Pig garden—compost pile	*L. monocytogenes* and *L. innocua*	NT
Pig garden—whey tank area	*L. innocua*	
Dog run	*L. monocytogenes*	NT
Pig pen water	*L. innocua*	
Pig pen dirt	*L. monocytogenes*	Yes
Pig pen bedding	*L. monocytogenes*	Yes
Chicken coop floor—dirt	*L. monocytogenes*	NT
Chicken run—old flooring	*L. monocytogenes*	NT
Chicken run walkway	*L. monocytogenes*	NT
Outbreak B samples		
Hydrated mould culture	*L. seeligeri*	
Finished water (UV treated/filtered from inside plant)	*L. seeligeri*	
Cistern pipe	*L. monocytogenes*	Yes
Pond water	*L. seeligeri*	
Lagoon water	*L. innocua*	
Sewage water	*L. monocytogenes*	NT
Cow feces	*L. innocua*	
Cow feed greens	*L. monocytogenes*	No
Water/rag in milk house	*L. monocytogenes*	Yes
Swallow nest	*L. monocytogenes*	Yes
Chicken feces	*L. ivanovii*	
Pheasant feces	*L. monocytogenes*	No

[1]NT, not tested.

L. seeligeri, *L. innocua*, and *L. ivanovii* (Table 3). *L.m.* isolates recovered from a cistern pipe, a rag soaked with water in the milking house, and a swallow's nest sample matched the outbreak *L.m.* strain PFGE profile. HPC and TC results in cistern water were 2.01 $\log_{10}$ CFU per mL (HPC) and 1.0 $\log_{10}$ CFU per 100 mL (TC) and in post-UV water, 2.47 $\log_{10}$ CFU per mL (HPC) and 0.30 $\log_{10}$ CFU per 100 mL (TC).

3.3. Investigation Results. A review of the raw milk sources, pasteurization procedures, records, and equipment in both plants A and B did not reveal any obvious food safety hazards that could lead to *L.m.* contamination of finished products (Table 1). Both plants appeared clean and well maintained. The detailed investigation of plant A, however, did reveal potential issues with ingredients, equipment sharing between raw and RTE food areas, and the proximity of the farm animals to the plant. The detailed investigation of plant B suggested issues with the water supply, which were then further investigated.

3.3.1. Plant A Investigation. In plant A, temperature records for raw milk receipts ranged from 0.8°C to 5.6°C, with acidity levels within normal range (pH 6.6 to 6.8). Once a week, 400 litres of raw goat's milk from a licensed dairy farm was used to make ~40 kg of cheese, and once every two weeks, 400 litres of raw cow's milk from the provincial dairy pool was used to make ~40 kg of cheese. All raw milk was vat pasteurized at 63.3°C for a minimum time of 30 minutes. Phosphatase tests of 11 different cheese types and production dates were negative, confirming that the milk used to make the cheese was properly pasteurized. The pasteurization equipment was tested to verify the accuracy of thermometers and timing clock. The integrity of vat jackets and the integrity of vat pasteurizer outlet protection valves were examined to

ensure the absence of leakage. Raw milk cross-contamination into pasteurized milk was assessed during transfer of pasteurized milk to cheese vats. The potential for cross-contamination via operator (hands or clothing), equipment (used for both raw and pasteurized milk), and splashing was also evaluated. The operator demonstrated good understanding of the risks of cross-contamination, and hand/apron sanitizing was frequent. One piece of equipment, the pH meter, was found to be shared by raw and pasteurized milk sources. The probe was rinsed but not sanitized between testing of raw milk and pasteurized cheese curds. Testing of pH probe buffer solutions did not detect *L.m.* The probability of the pH probe and/or the pH probe buffer as a source of cross-contamination was assessed as unlikely (neutral investigation finding noted in Table 1). All areas of the dairy processing plant not in contact with contaminated cheeses or spray culture solution bottles were negative for *L.m.* In addition, *L.m.* and other *Listeria* spp. were detected in the environment outside plant A. This suggested a likely *L.m.* dissemination from the farm environment to the dairy processing plant, further supported by the finding that toilet facilities were shared between farm workers and dairy plant employees. However, a direct link to the farm through an animal vector could not be definitely established, as farm samples were collected several weeks into the investigation and it was discovered that during this time the operator had fed the recalled *L.m.* contaminated cheese to farm animals (i.e., pigs, housed next door to free-range chickens).

The interior of the dairy processing plant environment appeared clean, sanitary, and well maintained based on a visual inspection. Approved food grade sanitizers designed for use in a food processing environment were correctly employed, verified by a review of the sanitation records. Environmental sampling of the plant interior revealed only five of 40 (12.5%) swabs positive for *L.m.*, all from within the aging room (Table 2). Unacceptable investigation findings were found in the handling of two spray cultures in the aging room. A *Penicillium* mould culture solution used to spray the outer surface of the camembert soft mould-ripened cheese, and a *Brevibacterium* culture solution used to spray the outer surface of tommes bacterial smear-ripened cheese for fermentation were used for flavor and creation of a consistent rind on the outer surfaces of the cheeses. Preparation of solutions required rehydrating of freeze dried commercial culture that was added to boiled and cooled water containing 3% salt. The *Brevibacterium* culture solution also contained one part of commercial beer. Hydrated cultures were then stored in plastic spray bottles on a shelf in the aging room. However, these bottles were not regularly washed or sanitized. Further, new solution was added to the existing solution so that older solutions were not emptied out when bottles were refilled. The operator had typically stored the spray culture solution bottles in a 4°C refrigerator. However, this refrigerator malfunctioned three months prior to the outbreak, and the bottles were subsequently stored on a shelf in the aging room (kept at 10°C). The operator could not recall when the bottles had been last emptied, washed, and sanitized, describing this as occurring several weeks or months prior. Further, a worker occasionally prepared these solutions who had not received any training in dairy plant processes. As not all personnel working in the dairy processing plant were trained or licensed as dairy plant process workers, unsanitary handling of ingredients and equipment and unsatisfactory hygiene practices were also considered plausible causes of cross-contamination within the plant. Dairy processing plant entry access was also reviewed. Before personnel proceeded into the plant, entry was controlled by having personnel change clothes in a designated changing room area, put on dedicated plant shoes, and wash and sanitize hands in a sink in an adjacent toilet. However, the sink and toilet were shared with nondairy plant workers engaged in activities on the hobby farm, which included animals.

3.3.2. Plant B Investigation. In plant B, records for August 16 indicated that the raw milk used to make the implicated batch of cheese was at 2.2°C prior to pasteurization. The pH of the milk was normal (pH = 6.8). SPC tests of the raw milk were performed twice per month, with a previous year annual average of 3.09 $\log_{10}$ CFU/mL. The raw cow's milk was supplied from the dairy processing plant's own licensed dairy farm located on the same site, and a daily production yield of 750 L of cow's milk was processed into several varieties of raw milk and pasteurized milk cheeses three times per week. Similar to the investigation of plant A, no issues were found with the pasteurization equipment or with the pasteurization method. Milk was vat pasteurized above the minimum pasteurization time and temperature to 65°C for 31 minutes. Further, no issues were identified to indicate any postpasteurization contamination of milk from raw milk (splashing or entering). The operator was also aware of potential cross-contamination issues. Raw milk and pasteurized milk cheese were not produced on the same day, and cleaning and sanitizing were performed at the end and beginning of each production. One of two dairy processing plant workers was responsible for milking cows, and the work duties, habits, and sanitary procedures of this worker were assessed as satisfactory. Clothing specific to the milking operation was put on over street clothes in the milk house. When called to work at the plant, the worker first washed hair and hands in the dairy farm milking house, removed clothing, and put on clean street clothes. At the plant office, street clothes were removed, and clean dairy clothes were put on before entering the dairy processing plant. Upon entering, rubber boots, apron, and hair net were worn (all used exclusively in the plant); then hands were washed and sanitized before proceeding into the processing room. A visual inspection of the processing areas did not reveal any deficiencies in the cleaning and sanitation program. These inspection findings were supported by environmental sampling, with none of the 22 swabs of food contact and nonfood contact surfaces within the plant testing positive for *Listeria* spp.

However, the ingredients used to manufacture cheese were not acceptable. Several ingredients were added to milk or cheese postpasteurization including freeze dried starter cultures, vegetable rennet, salt, natamycin, calcium chloride, annatto coloring, and hot water (66°C) to wash curds. During initial testing of 31 ingredients, only one ingredient (3.2%), a hydrated mould culture solution used on September 16 tested positive for *Listeria* spp. (*L. seeligeri*). Subsequent testing of all

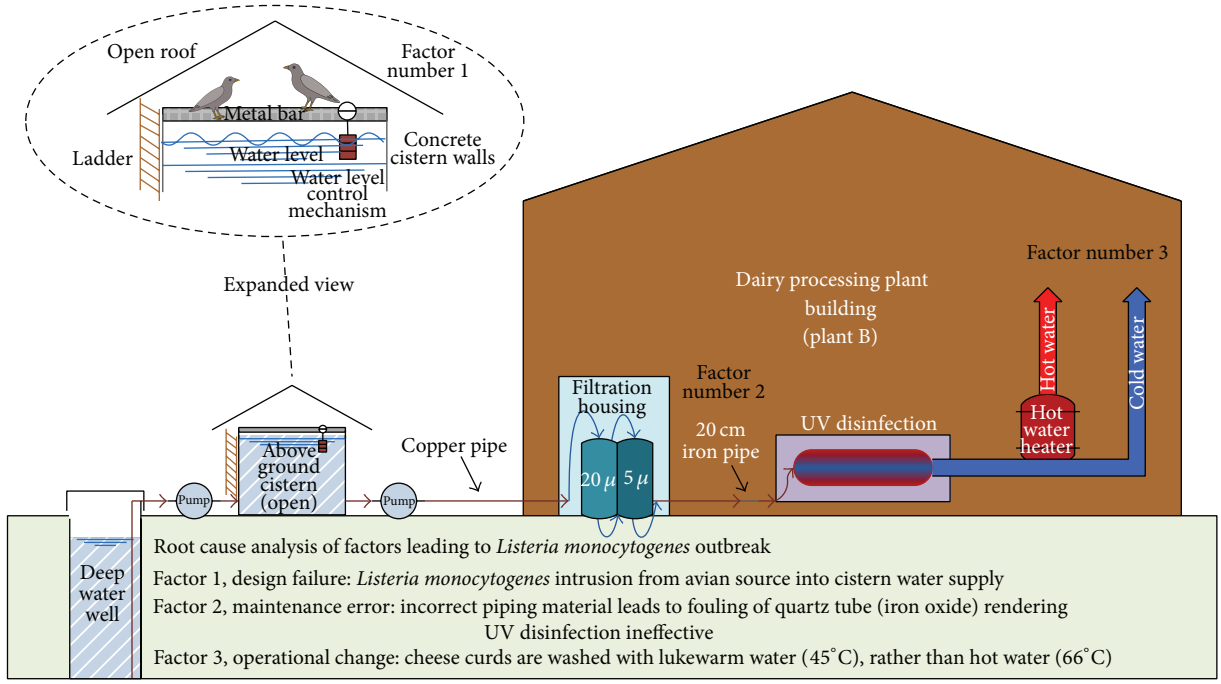

Figure 2: Schematic diagram of water supply system in dairy processing plant B.

cultures and ingredients used in the hydrated solutions was negative, including an initial water test. An examination of the plant's production records revealed a critical anomaly: during the August 16 production of SRC, warm water at 45°C (a mixture of hot and cold water) was used to wash cheese curds, rather than the plants' normal procedure of using hot water at 66°C. Curd washing required the addition of 50 to 75 L of water to the cheese vat. This led to a hypothesis that the water used in the plant may have been the source of *L.m.*, and the investigation focused on the water supply. The initial test of in-plant water (100 mL was taken from the water hose in the plant used for curd washing) was negative for *L.m.* and other *Listeria* spp. When a larger sample of 1.5 L, rather than 100 mL, of tap water was examined on repeat sampling, *L. seeligeri* was found in the second test.

Water for Plant B was supplied by a private deep well located several hundred metres away (Figure 2). Well water was pumped to an aboveground open concrete cistern located 150 m from the dairy processing plant. Although water samples from the cistern were negative for *Listeria* spp., total coliform and HPC results of cistern and post-UV treated water were unsatisfactory. The colour of the water in the cistern was elevated at 70 true colour units (TCU). However, an environmental swab of bird droppings on a pipe directly above the water surface in the cistern was positive and matched the PFGE profile of the outbreak *L.m.* strain. Other faecal and environmental samples in and around the farm revealed several *Listeria* spp., including matches to the outbreak strain in swabs taken from a rag regularly soaked with water in the milk house and in a barn swallow's nest (Table 3). From the cistern, water was pumped to the dairy processing plant and then through successive filters of 20 μm and 5 μm before passing through a UV water sterilizer into the plant. Further investigation of the water supply revealed a recently repaired section of piping supplying water to the UV sterilizer, where a 20 cm (8 inch) piece of iron pipe was spliced into the existing copper line. When the UV sterilizer was disassembled, a build-up of debris, suspected to be iron oxide, was found on the quartz sleeve that separated the water from the UV bulb. An engineer consultant calculated a UV transmittance of 47.5% based on the UV absorbance of the filtration unit (0.323 au) and on the elevated colour of the cistern water (with an unfouled quartz sleeve), a value considered extremely low. The combination of excessive cistern water colour and the fouled quartz sleeve would further lower the calculated UV transmittance, rendering the UV sterilizer ineffective.

4. Discussion and Conclusion

Postpasteurization contamination of SRC occurred in both outbreaks. Neither pasteurization failure nor contaminated milk supply were likely contributors to the outbreaks. Both dairy processing plants were visually very clean, and inspection observations found acceptable sanitation levels in the interior plant environments. These observations were supported by environmental swab tests of food contact and nonfood contact surfaces in the plants. In plant B, no swab samples were positive for *Listeria* spp., and in plant A only five surfaces, all in one room, were positive for *L.m.* However, inspections revealed that the external environments of both dairy processing plants were either neutral or unacceptable, observations also supported by test findings of high *Listeria* spp. and *L.m.* prevalence in areas outside the dairy processing plant and in the farm environment. Outbreak A events likely

resulted from a GMP procedural failure arising from incorrect handling practices of culture spray solutions, while outbreak B was attributed to a multibarrier failure in the potable water supply to the plant. Contamination of the water held in the unprotected water cistern, failure in the UV water disinfection system, and subsequent addition of contaminated water in the curd washing step led to contamination of the SRC. Plant investigations and laboratory testing data identified barn swallows as the environmental reservoir and source of the *L.m.* in outbreak B. In outbreak A, while the source of the contamination was successfully traced to culture spray bottles and the plant's cheese aging room, whether the *L.m.* came from environmental sources outside the dairy processing plant could not be confirmed. Although a direct link could not be established, the most likely cross-contamination point was the shared toilet between farm and dairy processing plant workers, suggesting a potential for *L.m.* transmission from outside sources into the plant as *Listeria* is readily found in soil and farm environments. In both outbreaks, we posit that environmental transmission of very low numbers of *L.m.* was introduced during postpasteurization steps into SRC and other types of cheese, allowing growth of *L.m.* to very high numbers capable of causing illness.

As *L.m.* is a particularly cold-tolerant organism, very low numbers of *L.m.* may have initially contaminated the spray solutions in outbreak A. Over several months, we hypothesize that *L.m.* grew in the spray culture solution bottles, and when sprayed onto the cheeses, it further multiplied in the contaminated SRC. Processing of more than one variety of cheese likely resulted in cross-contamination among the types of cheese, with potential routes being from personnel handling the contaminated *L.m.* positive cheeses, from handling the spray culture bottles, and/or from *L.m.* on personnel clothing. A possible *L.m.* transmission route into the plant could be from individuals doing farm work, such as handling manure and garden dirt, leading to the contamination of shared toilet facilities and change room areas in the plant, though this theory is only speculative. While the outbreak *L.m.* strain was detected in several hobby farm samples, we could not confirm the animals or farm as the direct source, as *L.m.*-contaminated cheese recalled during the outbreak investigation had been fed to the pigs.

In outbreak B, the findings support a point-source contamination event that affected a single lot of SRC. The intensive investigation of the water source may not have occurred if the operator had not saved the September 16 hydrated culture solution. Following outbreak A and prior to outbreak B, the BCCDC had implemented a new directive to all operators to empty, clean, and sanitize hydrated culture solution bottles after use. However, out of prudence and concern, this solution was purposefully saved for subsequent testing by the operator of plant B upon notification of the recall and illness. A follow-up interview of the operator revealed that plant water normally used to wash curds was used to make the culture solution, instead of the recommended method of preparing the solution with boiled and cooled water.

Through a root cause analysis, it was revealed that the factors contributing to outbreak B included a design failure, a maintenance error, and an operational change (Figure 2). Individually, these factors would not have led to the cheese contamination event that resulted in a listeriosis outbreak. However, when combined, these factors created conditions that allowed *L.m.* into the water supply, its survival due to inadequate water treatment, and its transmission into the food during postpasteurization cheese processing. Focused investigation into the water supply revealed multiple issues: (1) the cistern was open to birds, and barn swallows were observed to sit on the metal bar directly above the surface of the water, drink from the cistern water supply, and defecate on the bar and into the cistern; (2) dried bird droppings were also observed on the upper lip of the cistern, and droppings were collected from the metal bar directly above the water located in the cistern; (3) the colour of water measured in the cistern was found to be elevated; and (4) an investigation of the UV disinfection system, once disassembled, revealed fouling of the quartz tube, likely from iron oxide, from the recent repair and splicing of a section of iron pipe into the water supply line. This resulted in minimal microbial reduction in water treated with this UV disinfection system. This assessment was supported by the HPC results, with 65% more bacteria found in the treated versus untreated (cistern) water supply.

Although it is not known how long the UV disinfection system was failing, hot water (66°C), normally used to wash the curds would likely have killed any *Listeria* organisms that had survived the faulty UV water sterilizer. The August 16 lot code of SRC is suspected to have been contaminated with *L.m.* from the in-house treated water supply. On this date, only warm water (45°C from a mixture of hot and cold water), not hot water, was used to wash the curds. While washing curds with warm water is a normal and acceptable practice in dairy processing plants, we suspect that addition of warm water to the curds allowed the introduction of viable *L.m.* into the cheese curd from the water supply. The original source of the *L.m.* in the water supply pointed to barn swallows as the outbreak strain of *L.m.* was found on the pipe in the cistern containing bird droppings, in a barn swallow's nest in the farm area, and on a water rag used in the milking house (Table 2).

Another interesting finding from outbreak B was the unusual illness presentation, with only noninvasive listeriosis cases observed. *L.m.* was detected in clinical samples of stool (faeces). In the year of the outbreak, 2002, neither invasive nor noninvasive listeriosis were nationally nor provincially notifiable diseases. After the two 2002 listeriosis outbreaks in BC, invasive listeriosis disease became a reportable condition in the province of BC [58]. Nationally, invasive listeriosis became a reportable condition in 2007, although noninvasive cases are still not reportable or tracked in Canada, including BC [58, 59]. We report here a very rare event where a noninvasive *L.m.* outbreak was discovered and for the first time reported in BC and Canada. Findings from the two BC outbreaks described here and other provincial outbreaks were shared with the federal authorities and collectively led to improvements in the Canadian listeriosis reference services through offering of enhanced and prompt PFGE testing, creation of standardized food histories, and recommendations for testing for *L.m.* in cases of noninvasive febrile gastroenteritis, when other pathogens were not detected in stool [60].

Immediate recommendations made by BC provincial authorities included a requirement for the two dairy processors involved in the outbreaks to test for *L.m.* before releasing their products for sale, and a new requirement for periodic industry funded testing of SRC products for *L.m.* from other provincial dairy processing plants. All dairy processing plants were required to submit current HACCP-based or equivalent food safety plans, provide product lists for their operations, demonstrate water used as an ingredient meets requirements for potability, and ensure that effective physical barriers exist between their plants and other agricultural uses. Plant B was also required to bring in water to be held in a closed containment system for processing. Further recommendations for dairy and public health inspectors were to conduct a review of all private water systems supplying potable water to dairy processing plants, include the management of ripening solutions under the HACCP procedures for plants, incorporate additional materials into the dairy worker course, regularly collect environmental swabs for *L.m.* testing, and conduct annual auditing of SRC products during inspections.

Inspections of dairy processing plants did lead to industry improvements and a reduction in the numbers of listeriosis cases in the years following the 2002 outbreaks. In a 2009 survey of dairy, meat, and fish processors in BC, no dairy products nor processing food contact surfaces in dairy processing plants were found to contain *L.m.* or other *Listeria* spp. [61]. No illnesses linked to SRC produced in dairy processing plants under provincial inspection authority have been detected since 2002. Routine inspections, however, have occasionally detected *L.m.* in cheese and environmental swabs. Noncompliant food and environmental swab test results and noncompliant observations during inspections have led to both product recalls and incremental improvements in dairy processing plants when deficiencies noted on inspections are addressed (unpublished data). We believe that these interventions, which arose from direct inspection observations and sample testing, have contributed to the prevention of illness and are necessary for public health.

In summary, investigations of foodborne outbreaks can be complex, requiring multiagency support, and extensive on-site inspection before the root cause of pathogen contamination of manufactured foods can be established. Specifically, in the outbreaks reported here, environmental sampling assisted in focusing on the inspections, generating hypotheses, and formulating the questions asked of plant operators during follow-up inspection interviews. *L.m.* transmission into cheese was uncovered during subsequent operator interviews and investigations. The complexity of the investigations required coordinated response from multiple experts, including dairy and health inspectors, epidemiologists, engineers, laboratory technologists, agrologists, and physicians.

Inspectors and regulators responsible for oversight of manufacturing processes require detailed systems knowledge to understand where errors can occur. Many regulatory agencies are moving towards outcome based guidance, reliance on inspection of records, and compliance with record keeping. In outbreak B, there should not have been an outbreak when the dairy worker washed the curds with lukewarm water. The issue was that the water should have been potable, and it was not. This dairy had a secondary UV water disinfection system, supported with monitoring records to show that the system had been operating normally and had been maintained as required by the system manufacturer. From a regulatory and records perspective, the dairy was in full compliance. The problem lay in the source water contamination (cistern was open to animals) compounded by a recent improper repair to the water line. The multiple factor failures illustrate how events can lead to illness, despite compliance with regulations and despite good records. A surface examination of records would not have revealed these problems. We are concerned that with regulatory agencies now moving towards a model of records inspection concomitant with a reduced inspection frequency in manufacturing settings could potentially lead to missed opportunities for detecting and correcting errors that are often found during physical and process inspections.

In addition, findings from these listeriosis outbreaks demonstrate the importance of adhering to strict processing procedures to minimize the survival and spread of *L.m.* during postpasteurization product handling and that environmental transmission of *L.m.* into foods can occur from wild animal sources. Further, considering that products implicated in the outbreaks were prepared from pasteurized milk and that these products may be erroneously considered safe for consumption by populations vulnerable for listeriosis [30], we recommend that pregnant women and immunocompromised and elderly populations >65 years old should avoid the consumption of pasteurized or unpasteurized SRC. This recommendation is consistent with relative susceptibility risk of these populations to listeriosis and with the advice given by the federal government of Canada [62, 63]. Ongoing vigilance from food manufacturers and public health inspectors are necessary to limit opportunities for harmful bacteria to enter the food supply. As *Listeria* is ubiquitous in the environment, special precautions are recommended for dairy processing plants and other food processing plants located adjacent to farms and wildlife animals.

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

The Water Cycle, a Potential Source of the Bacterial Pathogen *Bacillus cereus*

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The behaviour of the sporulating soil-dwelling *Bacillus cereus sensu lato* (*B. cereus sl*) which includes foodborne pathogenic strains has been extensively studied in relation to its various animal hosts. The aim of this environmental study was to investigate the water compartments (rain and soil water, as well as groundwater) closely linked to the primary *B. cereus sl* reservoir, for which available data are limited. *B. cereus sl* was present, primarily as spores, in all of the tested compartments of an agricultural site, including water from rain to groundwater through soil. During rain events, leachates collected after transfer through the soil eventually reached the groundwater and were loaded with *B. cereus sl*. In groundwater samples, newly introduced spores of a *B. cereus* model strain were able to germinate, and vegetative cells arising from this event were detected for up to 50 days. This first *B. cereus sl* investigation in the various types of interrelated environments suggests that the consideration of the aquatic compartment linked to soil and to climatic events should provide a better understanding of *B. cereus sl* ecology and thus be relevant for a more accurate risk assessment of food poisoning caused by *B. cereus sl* pathogenic strains.

1. Introduction

The life cycles of many human pathogens, including foodborne pathogens, comprise stages in the environment outside the eukaryotic host, for which data are sometimes limited but are required to monitor adverse effects on human health [1–3]. Ready-to-eat processed foods are usually a combination of multiple ingredients, each bringing its own possibility of contamination by a pathogen into the final food products. The bacterial endospores are among the most resistant forms of living organisms. Their resistance favors their survival to food processing. The multiplication of the vegetative cells formed after spore germination can occur in a wide range of temperatures, pH, or water activities and be the cause of foodborne poisonings [4].

The ubiquitous *B. cereus* group of bacteria, also called *Bacillus cereus sensu lato* (*B. cereus sl*), displays a broad diversity of phylogenetically related strains [5, 6], most of which harbour pathogenic characteristics [7–9]. This group includes closely related species, such as the causative agent

of anthrax *B. anthracis*, the entomopathogenic bacterium *B. thuringiensis*, or the human pathogen *B. cereus sensu stricto* (*B. cereus ss*), which is responsible for various human infections (septicaemia, endophthalmitis, periodontitis, etc.) [6, 10]. More importantly, *B. cereus ss* is a common source of foodborne poisoning, displaying emetic or diarrheal syndromes [10, 11] and representing 1 to 33% of cases of foodborne poisonings depending on countries [12]. A study conducted on *B. cereus ss* food poisoning outbreaks in France revealed the emergence of particularly virulent strains and emphasized the danger for public health caused by these bacteria [13], particularly in processed food that is not conserved in appropriate conditions. The sporulating ability of *B. cereus sl* allows these bacteria to survive in various stressful environments, which contributes to their ubiquity [14]. Spores highly resistant to heat and chemical and other stresses can adhere to stainless steel in the food industries despite cleaning-in-place procedures. Accordingly, these properties favour *B. cereus sl* contamination in processed food [14].

Moreover, *B. cereus sl* occurrence has been widely reported in a broad range of environments, such as many types of soils, sediments, dust, and plants (see [8–10, 15] for reviews). Despite increasing numbers of studies of microbiota in atmospheric environments (including water in clouds, fog droplets, etc.) that have regularly identified isolates of *Bacillus* spp. [16–18], *B. cereus sl* has not been specifically described. *Bacillus* occurrence in other natural habitats, such as aquatic environments, is the concern of only a limited number of studies. *B. cereus sl* has been rarely detected in surface fresh water samples [19]. In sediments of an Italian river, two of 83 isolates of sulphite-reducing bacteria were identified as *B. cereus sl* [20]. A few antibiotic-resistant isolates of *B. cereus sl* were also isolated in Chinese aquaculture environments [21]. These scant studies performed in aquatic environments confirm *B. cereus sl* ubiquity. To our knowledge, no specific investigation of *B. cereus sl* in groundwater has yet been performed. Groundwater is a common supply for drinking water, but groundwater is also an important source for irrigation, by flooding (e.g., grassland), by sprinkling (e.g., crops or vegetables), or by drip irrigation (e.g., orchards). If groundwater were contaminated with human pathogens, this practice could disseminate them to cultivated plants.

Despite its distribution in a broad range of natural habitats, little is known concerning how *B. cereus sl* bacteria behave in the environment. Most studies have focused on *B. thuringiensis* because this species is widely spread in the environment as a biological agent for pest control [15, 22] or on *B. anthracis* to elucidate how this species can lead to zoonosis [23]. However, ecological studies of *B. anthracis* have primarily focused on the conditions that lead to transmission of anthrax rather than on the fate of the cells in such environments [24, 25]. For other *B. cereus sl* bacteria, including the human pathogen *B. cereus ss*, many questions remain unanswered: it remains unclear whether *B. cereus ss* grows in bulk soil (whatever its physicochemical properties and nutrient availability) or whether *B. cereus ss* survives in the soil only as dormant spores until particularly favourable microenvironments (niches) are encountered to allow its growth [26]. Growth of *B. cereus sl* in such niches has previously been described, for example, in rhizosphere [27], insect cadavers [15], nematodes [28], arthropod gut microbiota [29], and earthworm guts [30, 31]. In a given environment, there may be a continuous flow of *B. cereus sl* strains with the arrival of new strains originating from elsewhere, whereas others disappear by death or by leaching. Soil is considered a reservoir for *B. cereus sl*; however, soil is not a static medium. Soil is permanently affected by water movement, which redistributes solutes, nutrients, and the geochemical background [32]. Little is known concerning the role of soil water flows during rain events in modifying the pool of *B. cereus sl* strains in soil or concerning the putative transfer of these bacteria to deeper environments and in their fate in groundwater.

A better understanding of *B. cereus sl* ecology should be useful for uncovering the still largely unknown determinants enabling the dispersal and fate of such populations in environmental conditions and might be relevant for a more accurate risk assessment of foodborne poisoning caused by *B. cereus sl* pathogenic strains. The aim of this study was to determine the occurrence and the fate of *B. cereus sl* from rain to groundwater through soil and to investigate a possible bacterial transfer from soil to water compartments. For this purpose, we sampled and isolated *B. cereus sl* in different soil and water compartments of the atmosphere-soil-groundwater continuum in an agroecosystem and performed *B. cereus ss* inoculation survival experiments of soil and water sampled in the same site.

2. Materials and Methods

2.1. Experimental Area. The experimental area was in south-eastern France at INRA-Avignon (altitude ASL 30 m, lon. 04°53′E, and lat. 43°55′N) 2 km north of the Durance River. The climate is Mediterranean, with a primary wet period in autumn (high intensity storm events) and with severe drought and high temperatures in summer. The various crops grown in this agricultural area require much irrigation water. The soil is composed of clayey silt (2.5 m maximum thickness) overlying an alluvial aquifer consisting of gravels and of sands (7 m depth) on marl bedrock. The water table depth ranges from 4 to 5.5 m [33]. The experimental area (conventional field of 0.6 ha, cultivated in 2010 with sunflower, in 2011 with durum wheat, and in 2012 with corn) is instrumented with 14 piezometers and with one lysimeter. Sampling was performed between April 2011 and April 2012.

2.2. Soil. The bulk soil used in this study was collected from the experimental field, as previously described [34], during the intercropping season, several months after the last harvest (June 23, 2011). The soil is a fine textured calcareous silty clay loam (Table 1). Soil samples were used in two approaches: occurrence studies and bacterial inoculation tests.

For *B. cereus sl* occurrence studies, the soil samples were collected in October 2011 from the 0–2, 2–10, 10–20, and 20–30 cm layers of the field and in April 2012 from the 0–2, 2–10, 10–20, 20–30, 30–60, and 60–90 cm layers of the field (Table 2). Surface soil samples (0–2 cm depth) were

Table 1: Selected physicochemical characteristics of the soil sampled (0–10 cm depth) for *B. cereus sl* inoculation experiments.

Soil physicochemical characteristic	Value
Clay ($g\,kg^{-1}$)*	323
Silt ($g\,kg^{-1}$)*	259
Sand ($g\,kg^{-1}$)*	45
Water hold capacity (% moisture content)	31.4
pH (water)	8.51
$CaCO_3$ ($g\,kg^{-1}$)	347
Cation-exchange capacity ($cmol\,kg^{-1}$)	11.4
Total organic carbon ($g\,kg^{-1}$)*	13.2
Total organic matter ($g\,kg^{-1}$)*	22.8
Total nitrogen ($g\,kg^{-1}$)*	1.54
$C:N$ ratio	8.59

* After decarbonatation.

collected with a cleaned stainless steel trowel; the other soil samples (<2 cm depth) were vertically collected by coring with a cleaned stainless steel auger. Soil samples were placed in clean plastic bags and stored at 4°C before *B. cereus sl* measurements (performed within 7 days). Substrate-induced respiration (SIR) with glucose was performed using the MicroResp technique [34], which is a rapid microtiter plate method to measure carbon dioxide evolved from carbon substrate amendments to determine the physiological profiles of soil microbial communities by using whole soil. The SIR microbial measurement is considered a good indicator of the total physiologically active bacteria in soil, which is a proxy of the active microbial biomass [35]. The data of *B. cereus sl* occurrence in soil were analysed using Student's t-test to determine P values for differences in expected values versus actual values.

For *B. cereus ss* inoculation experiments, the soil samples were collected in March 2012 from the 0–10 cm layer of the field, air-dried, and sieved into a size fraction between 2 and 3 mm. After sampling, drying, and sieving, the soil was slowly moistened and maintained at 21% of the gravimetric water content (a value equivalent to the water content usually observed in this soil at this time of the year and also optimal for microbial activities) and stored for seven days at room temperature before inoculation experiments [36].

2.3. Rainwater. Rainwater was collected on the site as follows: when rain was about to fall or when the rainfall event had recently begun, an autoclaved plastic bag (50 L) was placed in a circular 21 L bucket with a diameter of 0.30 m (corresponding to a surface area of $0.071\,m^2$). The bucket was held on a stick 2 m above the ground to avoid any risk of contamination by rain splashing on the soil. The bucket was in the middle of the experimental field, 25 m from the closest hedge to limit the risk of contamination by neighbouring vegetation. The rainwater was collected 3–18 h later and immediately filtered on a 0.2 μm pore size membrane. Then, the particles, including putative bacteria located on the filter, were suspended in 2 mL of filtered rainwater, giving a 100–200-fold concentration of the rain sample, depending on the volume of rainwater collected. The rain samples used for *B. cereus sl* occurrence studies were collected in October 2011, November 2011, March 2012, and April 2012. During all of these rainfall events, the dominant winds were south-easterly.

2.4. Soil Percolated Water. A lysimeter placed in a monolith of undisturbed soil (1.8 m × 2.6 m surface area and 2.2 m depth) permanently exposed to natural climatic conditions was at the north-west end of the field-site. Cultivated identical to the nearby field, the lysimeter is dedicated to the measurement and calculation of soil water fluxes at the plot scale, with a continuous monitoring of hydrographs of water drainage events [37]. The bottom of the lysimeter was equipped with drains and with a fluxmeter for the automatic recording of the amount of percolated water (one automatic measurement for every 20 g of water drained). The lysimeter was constructed in 2003 and had since been under crop rotation identical to the field next to the experimental site. A device was set up to collect, in sterile containers, leachate samples drawn from the drains of the lysimeter for periods of several hours, depending on the percolated water fluxes. Percolated water collected during five rain events from April 2011 to April 2012 was concentrated as described above for rainwater (Section 2.3). The recorded hydrographs of drained water enabled the calculation of the mean water flow rate and the period of drainage (except for April 2011, for which hydrodynamic data were not available). The mean *B. cereus sl* flow during the sampling period was calculated.

2.5. Groundwater. Groundwater was collected as follows: a piezometer, which tapped the upper part of the saturated zone (depth 6 m) and was in the south-east of the area, was hand-sampled using an immerged pump after the complete renewal of the water in the piezometer. Groundwater, which was collected in a sterile plastic bag, was concentrated as described above for rainwater (Section 2.3). The borehole was sampled four times in autumn 2011 and in spring 2012. All water samples presented the same features: the pH ranged between 7.1 and 7.2; the temperature was equal to 16 ± 0.1°C; and electrical conductivity, characterising the mineralisation of groundwater, ranged between 654 and 658 μS/cm. Previous analyses of groundwater (~60 samples) in several boreholes near the study area from 2009 to 2011 highlighted the predominance of Ca^{2+} and HCO_3^- in solution. The concentration of organic carbon in groundwater, which may be present as a particulate or dissolved, ranged between 0.4 and 9.6 mg/L, with a mean concentration equal to 1.3 mg/L.

2.6. Strains and Growth Conditions

2.6.1. Isolation and Enumeration of B. cereus sl from Environmental Samples. For the isolation, identification, and counts of *B. cereus sl* CFU in these environmental samples with the presence of background microbiota, we used a selective medium recommended by food authorities. The Mossel medium (also called MYP for mannitol-egg yolk-phenol

Table 2: Selected physicochemical and microbiological characteristics of the soil layers sampled in this study.

Depth of soil sampling (cm)	October 2011		April 2012			
	SIR-microbial biomass (μg C g^{-1} soil)	Humidity (% g DW soil)*	SIR-microbial biomass (μg C g^{-1} soil)	Humidity (% g DW soil)	NO_3^- (mg kg^{-1} soil)	pH-water
0–2	90	5.5	202	3.7	83.9	8.29
2–10	67	15.3	150	16.7	88.9	8.44
10–20	56	16.2	163	17.4	89.7	8.52
20–30	36	16.7	165	18.6	73.4	8.38
30–60	ND	ND	22	20.1	61.9	8.46
60–90	ND	ND	10	17.8	51.5	8.61

*DW: dry weight.

red-polymyxin-agar), which was purchased from Biokar Diagnostics (Beauvais, France), allows the growth of *B. cereus sl* bacteria [38] and their identification as lecithinase-positive and mannitol-negative colonies. Because of the existence of rare lecithinase-negative or mannitol-positive *B. cereus sl* strains that were discarded in these conditions, our bacterial counts might be slightly underestimated [38]. We used 10% TSA medium (Trypticase soy agar, Difco) to count culturable aerobic heterotrophs after incubation for 48–72 h at 20°C.

Aliquots of 0.25 g of soil were supplemented with 1 mL of sterile saline water and vortexed vigorously for 30 s. The suspension was serially diluted 10-fold in sterile saline water, and 100 μL of each dilution was plated in triplicate on Mossel medium and incubated for 24 h at 30°C. The variability between triplicates did not exceed 0.26 log CFU, indicating that the bacterial counts were reproducible. For determining the *B. cereus* spore density, the samples were pasteurised for 20 min at 70°C to eliminate vegetative cells and then incubated on selective medium. These thermoresistant bacteria were counted as spore forms because only spores of *B. cereus sl* survive at this temperature.

2.6.2. Inoculation of Environmental Samples with a B. cereus ss Model Strain. For the inoculation of environmental samples, the R2SK model strain for human pathogenic *B. cereus ss* strains was used. This strain is a spectinomycin- (Spc-) resistant strain derived from the ATCC 14579 strain [39]. The minimal temperature of growth for this mesophilic strain is 10°C [40]. Growth was routinely performed at 30°C on Luria Bertani (LB) medium (Difco), unless otherwise stated. LB-Spc (275 μg/mL) was used for counting this strain after the inoculation of natural samples.

B. cereus R2SK spores, which were prepared on the FNA sporulation medium [41] for 7 days at 25°C, were used for the inoculation of natural samples. These spores were washed three times in cold sterile saline (0.9% NaCl) water, pasteurised for 20 min at 70°C to remove any remaining vegetative cells, and stored at 4°C until use.

B. cereus R2SK vegetative cells used for the inoculation of natural samples were grown in Gordon's medium containing soil extracts [42]; however, only the first soil infusion step was performed. The medium was inoculated with 1% of an overnight culture of R2SK strain, and growth was performed at 30°C with shaking (200 rpm) until $OD_{600\,nm} = 3.0$. Then, the cells were washed twice in sterile saline water at 20°C before soil or water inoculation.

Soil aliquots (0.25 g) introduced into sterile 2 mL screw-cap microtubes were inoculated with 20 μL of *B. cereus* R2SK vegetative cells to reach approximately 4 log CFU/g (a level below that of the natural *B. cereus sl* population in soil) and incubated at 25°C in the dark. Counting was performed over time as described above for the enumeration of the natural *B. cereus sl* population (Section 2.6.1); however, serial dilutions were plated in triplicate on LB-Spc. The first count was performed immediately after inoculation, and a second count was performed one hour later to confirm that no rapid changes in bacterial counts had occurred, which could be caused, for instance, by the dissociation of long chains of vegetative cells or of putative spore aggregates, as previously described [43]. To determine the spore content, the initial suspension was rapidly (<1 min) transferred at 70°C to a water-bath for 20 min as described above (Section 2.6.1).

Groundwater aliquots (100 mL) were inoculated with 1 mL of *B. cereus* R2SK spores or of vegetative cells, which were prepared as described above, and incubated at 12°C in the dark without shaking.

When required, populations of *B. cereus* R2SK that were below the detection threshold (10 CFU/mL) were arbitrarily set at 9 CFU/mL, as previously described [40].

2.7. Diversity of B. cereus sl Natural Isolates. Some isolates ($n = 3$) originating from rainwater that were defined as *B. cereus sl* bacteria after growth on Mossel medium were characterised, as previously described [44], using API50CH (BioMérieux), which is a kit that allows the determination of bacterial respiration on 50 different carbohydrates [45] to confirm that the bacteria did belong to *B. cereus sl.* In addition, sequencing of the *rrs* gene was performed after a PCR amplification on 5 randomly selected strains ($n = 3$ from rain and $n = 2$ from groundwater), as previously described [6]. Briefly, *rrs* genes were amplified by PCR using the primers 5-AGA GTT TGA TC(A,C) TGG CTC AG 3-(forward primer) and 5-GG(A,C) TAC CTT GTT ACG A(T,C)T TC 3-(reverse primer), using the following temperature profile: 30 cycles of

94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 5 min. The amplification products were purified and DNA-sequenced by Eurogentec. A BLASTN search was performed against nr/nt database to confirm that best hits (with 100% query coverage and 97–100% identity) were obtained with *B. cereus sl* sequences. These methods are powerful tools for identifying *B. cereus sl* but are not able to discriminate isolates at the species level.

The diversity of the *B. cereus sl* natural isolates was assessed using a fingerprint method (M13 PCR), as previously described [46, 47], on 80 randomly selected isolates collected from soil samples originating from various depths ($n = 40$) and from percolated water ($n = 40$). The images of the PCR fragments were analysed using the Biogen 99.04 software (Vilber Lourmat, Marne-la-Vallée, France) for band detection, binary matrix generation, similarity coefficient calculation, and dendrogram construction. Images were also sight-checked to clear unspecific bands detected by the software. Similar visual patterns were obtained between duplicates. A control strain (ATCC14579) was always used, which showed >85% identity in 15 independent analyses. Thus, genuine distinct isolates were distinguished from putative clones when the percentage similarities were <85%.

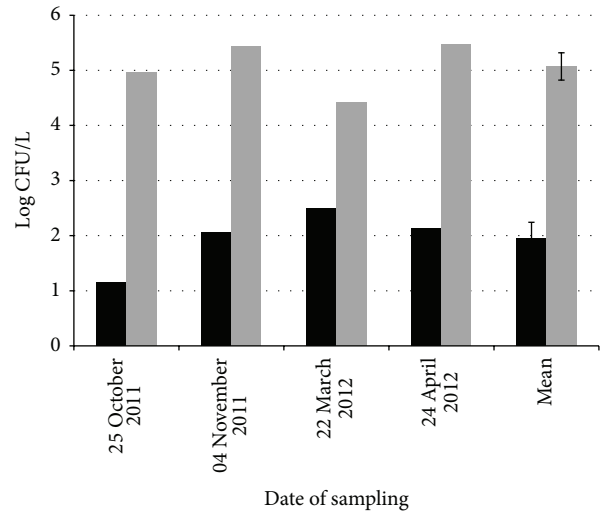

Figure 1: Bacteria isolated from rainwater. CFU counts were performed in four independent rain samples. The calculated mean values ± SEM from these four samples are also indicated. Grey bars: total culturable aerobic heterotrophs; black bars: *B. cereus sl* bacteria.

3. Results

3.1. Isolation of B. cereus sl Strains from Rainwater. The occurrence of *B. cereus sl* ubiquitous bacteria has never been studied in rain and was investigated in this study.

Our results (Figure 1) indicate that the level of total culturable bacteria ranged from 4.41 to 5.48 log CFU/L in the four rainwater samples (mean ± SEM 5.07 ± 0.25 log CFU/L). The investigated samples always contained culturable *B. cereus sl* at densities ranging from 1.16 to 2.50 log CFU/L (mean ± SEM 1.96 ± 0.28 *B. cereus sl* log CFU/L), 2 to 4 log lower than the total culturable bacteria. Both the phenotypic characterisation (using an API50CH kit) and sequencing of the *rrs* gene, which were performed on a few isolates, confirmed that the isolates were genuine *B. cereus sl* isolates.

3.2. Occurrence of B. cereus sl in an Agricultural Soil. B. cereus sl bacteria are commonly isolated from soils. The level of the natural culturable *B. cereus sl* population was investigated in the soil used during our experiments.

As expected, *B. cereus sl* bacteria were present in all of the soil samples, with a population ranging between 4.37 and 5.23 log *B. cereus sl*/g of soil (Table 3). The mean densities ± SEM per gram of soil of total *B. cereus sl* and thermoresistant *B. cereus sl* CFU (i.e., spores) were not significantly different (4.92 ± 0.09 versus 5.06 ± 0.04 log CFU/g, $P > 0.05$), indicating that *B. cereus sl* was almost exclusively present as spores in the soil tested.

The different seasons did not seem to modify the occurrence of the natural *B. cereus sl* population of this soil; the mean density ± SEM per gram of soil displayed no significant difference between samples collected in October and in April (5.09 ± 0.04 versus 4.92 ± 0.08 log CFU/g, $P > 0.05$).

In addition, the amount of *B. cereus sl* isolated did not seem to be influenced by the depth of soil in our sampling conditions. For instance, the mean *B. cereus sl* densities ± SEM per gram of soil were not significantly different between the superficial horizon (0–20 cm: 5.01 ± 0.06 log CFU/g) and the deeper depths (20–90 cm: 4.95 ± 0.09 log CFU/g, $P > 0.05$).

The decrease of values obtained using SIR measurements with the depth of soil indicates a higher active microbial biomass content in the upper layers of soil investigated (Table 2).

Altogether, these data suggest that the *B. cereus sl* population consisted nearly exclusively of dormant spores in the tested soil and was not affected by the season or by the soil depth (down to 90 cm) (Table 3).

3.3. B. cereus sl Isolated from Soil Leaching through the Lysimeter. B. cereus sl was isolated from all of the samples of water that percolated through the soil in the lysimeter; however, the density of its population and its estimated flux (mean during sampling period) varied (Table 4). We investigated thermoresistant *B. cereus sl* CFU (i.e., spores) in the three leaching water samples from 2012, and their densities were similar to those densities of the total *B. cereus sl* CFU (mean values: 3.76 and 3.69 log CFU/L, resp.), suggesting that *B. cereus sl* was almost exclusively present as spores in water soil leachates.

During the November 2011 rainfall event, six distinct fractions of the leachates were collected, three at the beginning and three at the end of the leaching event. The mean fluxes of *B. cereus sl* isolated during the beginning of the leaching event were significantly higher than during the end of the same leaching event (4.97 ± 0.39 versus 3.15 ± 0.27 *B. cereus sl* log CFU/L, mean ± SEM, $P < 0.05$, Student's t-test) (Table 4). At the time scale of a single rainfall event and at the megascopic scale of the lysimeter, the bacteria density or flux increases with the water flux of the drained water.

Table 3: Occurrence of *B. cereus sl* in an agricultural soil*.

Depth of soil sampling	October 2011		April 2012	
	Total *B. cereus sl* log CFU/g of soil	Thermoresistant *B. cereus sl* log CFU/g of soil	Total *B. cereus sl* log CFU/g of soil	Thermoresistant *B. cereus sl* log CFU/g of soil
0–2 cm	5.00	5.22	4.43	4.95
2–10 cm	5.05	5.23	4.91	4.95
10–20 cm	4.98	5.07	5.23	5.15
20–30 cm	5.16	5.02	5.02	5.06
30–60 cm	ND**	ND	5.04	5.08
60–90 cm	ND	ND	4.37	4.88

*Values from triplicate measurements of the samples (see Section 2).
**ND: not done.

Table 4: Lysimeter leaching events: *B. cereus sl* density in water percolated from soil. Water flows during sampling periods. *B. cereus sl* flows estimated during sampling periods.

Date of rain event	Rainwater (mm)	Dates of water sampling	*B. cereus sl* (log CFU/L)	Mean water flow during the sampling period (L/h)	Mean *B. cereus sl* flow during the sampling period (log CFU/h)
25-26 April 2011	33.5	25 April 2011	5.48	ND***	ND
01–04 June 2011	61.5	4 June 2011	5.69	0.698	5.53
		3 November 2011*	5.18	0.187	4.46
		3-4 November 2011*	4.98	0.551	4.72
		4 November 2011*	5.46	1.940	5.75
01–07 November 2011	183	9-10 November 2011**	3.95	0.266	3.38
		10–14 November 2011**	4.26	0.163	3.47
		14 November 2011**	3.48	0.134	2.61
03-04 April 2012	40	5 April 2012	3.62	0.049	2.31
		5-6 April 2012	3.66	0.027	2.09
10 April 2012	10	10–12 April 2012	3.83	0.006	1.61

*Measurements performed at the beginning of the November 2011 rain-leaching event.
**Measurements performed at the end of the November 2011 rain-leaching event.
***ND: not done.

3.4. B. cereus sl Isolated from Groundwater. Samples of groundwater were collected and investigated for the presence of *B. cereus sl* (Table 5). These samples were collected during a rainfall event (6 April 2012) or several weeks later (13 October 2011).

Three of the four samples indicated the presence of culturable *B. cereus sl*. In these three samples, the number of CFUs was equal to the detection threshold value, suggesting that only a low density of *B. cereus sl* was present in the studied groundwater samples.

3.5. Diversity of Environmental Isolates. To obtain an overview of the diversity of *B. cereus sl* natural isolates from soil samples, a fingerprint method was performed on 40 isolates (originating from various depths). To determine whether some groups of strains could be preferentially leached out from soil, the method was also applied on 40 isolates collected from percolated water (Figure 2).

High diversity was observed among the isolates of *B. cereus sl*, with many of them displaying different fingerprint profiles. Such diversity was observed for both soil and percolated water isolates. Among 80 isolates tested, at least 56 were considered distinct (displaying a similarity <85%). The dendrogram did not reveal any specific group of isolates from soil or from percolated water.

3.6. Behaviour of B. cereus ss in Aliquots of Soil and of Water. We monitored how a newly introduced model *B. cereus ss* population evolved over time in aliquots of soil and of groundwater that contained their natural microbiota. The water samples originating from rain and from percolated water were not investigated in this study because they were considered transient environments for *B. cereus sl*.

3.6.1. Behaviour of B. cereus ss in Aliquots of Soil. The natural *B. cereus sl* population of the soil used for inoculation

Table 5: *B. cereus sl* CFU isolated from groundwater.

Date of water sampling	*B. cereus sl* log CFU/L*	Previous rainfall event
13 October 2011	0.17 (0.17)	06 August 2011 (54.5 mm) 03 September 2011 (42 mm)
14 November 2011	0.96 (0.96)	01–07 November 2011 (163 mm)
06 April 2012	Below limit of detection (<0.96)	03–10 April 2012 (50 mm)**
18 April 2012	0.43 (0.43)	03–10 April 2012 (50 mm)

*The detection threshold is indicated in brackets and depends on the volume of water initially sampled.
**First significant rainfall event after several months (previous rainfall event in November 2011).

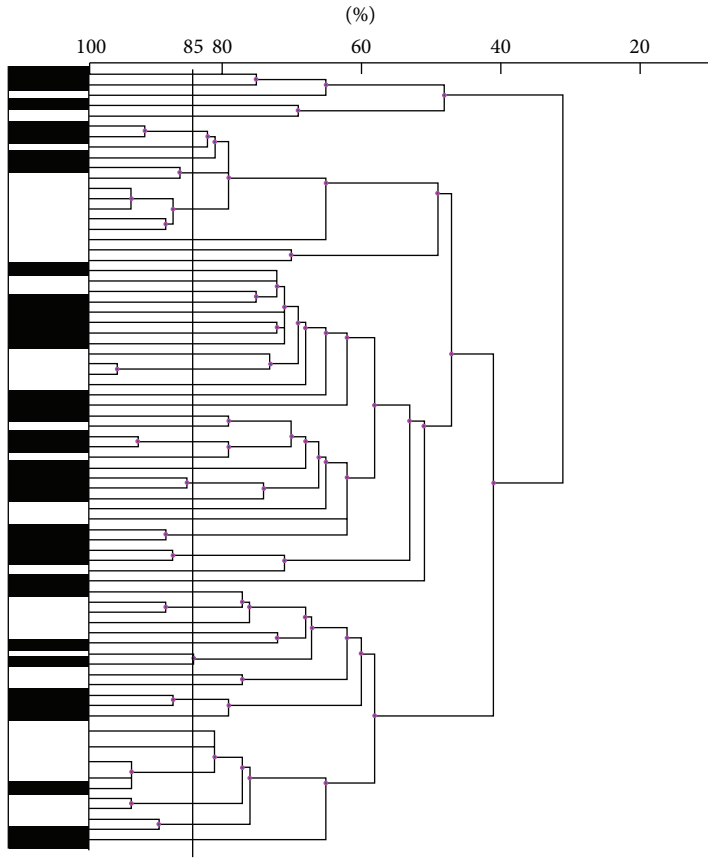

Figure 2: Dendrogram illustrating the diversity of *B. cereus sl* natural isolates. M13-PCR was performed on 40 isolates from soil (black boxes) and on 40 isolates from percolated water (white boxes). Genuine distinct isolates were distinguished from putative clones when the percentage similarity (indicated above) was <85% (black vertical bar).

experiments was 4.89 log CFU/g (mean value of duplicate experiments, range 4.82–4.94). Soil samples were inoculated by *B. cereus ss* vegetative cells of the Spc-resistant R2SK strain at a density between 3.66 and 4.16 log CFU/g, slightly below that of the natural *B. cereus sl* population level in this soil. A noninoculated control soil sample displayed no detectable Spc-resistant CFUs. Counts performed over time showed a rapid decrease of approximately 0.5–1 log in the newly introduced population during the first three days following inoculation (Figure 3). This decrease was concomitant with the appearance of thermoresistant *B. cereus* R2SK CFU (i.e., spores).

After this period, the remaining newly introduced *B. cereus* R2SK population was represented only by spores. This population reached a steady state, with no further changes in the level of *B. cereus* R2SK population observed up to 50 days of incubation at 25°C. Soil samples inoculated with vegetative cells (at 7.8 log CFU/g) of *B. cereus* R2SK still displayed high levels of thermoresistant *B. cereus* R2SK CFU (6.9 log CFU/g) after a 10-month period of storage in the dark at room temperature (not shown). This result indicates that the spores were extremely stable in this environment.

In addition, no major change in the level of the *B. cereus* R2SK population was observed over time when the soil was

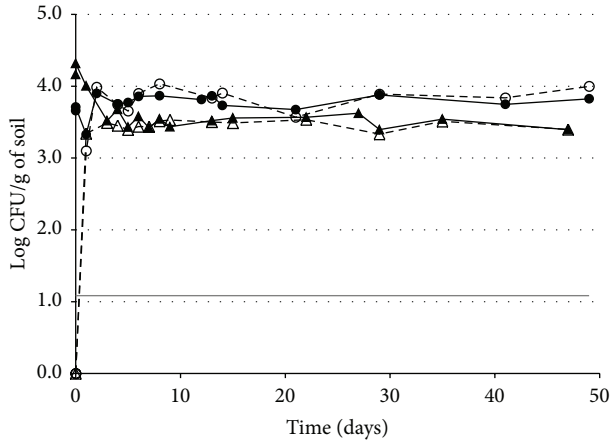

Figure 3: Fate of *B. cereus ss* vegetative cells introduced into the soil. Counts of CFUs and thermoresistant CFUs (i.e., spores) of *B. cereus ss* were performed over time. Two independent experiments (triangles, replicate 1; circles, replicate 2) are presented. Full symbols and full lines: *B. cereus ss* total CFU; open symbols and dashed lines: *B. cereus ss* thermoresistant CFU; grey horizontal line: detection threshold (1.08 log CFU/g). The data for the spores at time 0 were below the detection threshold but were arbitrarily set at 1 CFU/g (i.e., 0 log CFU/g) because growth conditions for the preparation of the inoculum were not appropriate to allow sporulation.

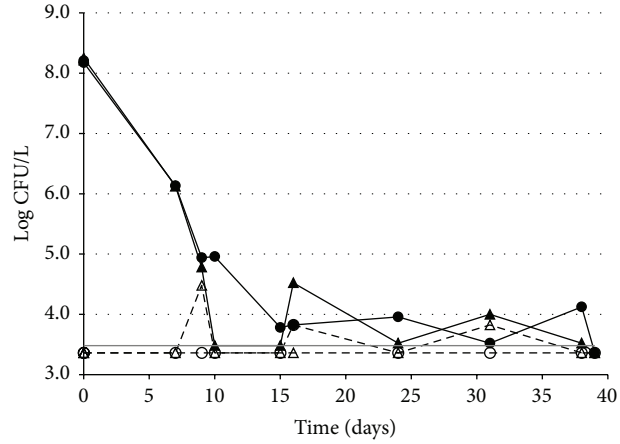

Figure 4: Fate of *B. cereus ss* vegetative cells introduced in groundwater. Counts of CFUs and thermoresistant CFUs (i.e., spores) of *B. cereus ss* were performed over time. Two independent experiments (triangles, replicate 1; circles, replicate 2) are presented. Full symbols and full lines: *B. cereus ss* total CFU; open symbols and dashed lines: *B. cereus ss* thermoresistant CFU; grey horizontal line: detection threshold (3.48 log CFU/L). The data below the detection threshold were arbitrarily set at 3.36 log CFU/L. (This value corresponds to the maximum CFU value possible below the detection limit.)

inoculated with spores instead of with vegetative cells (not shown).

3.6.2. Behaviour of *B. cereus ss* in Aliquots of Water

(1) In Groundwater Inoculated with Vegetative Cells. Aliquots of groundwater were inoculated with *B. cereus ss* strain R2SK vegetative cells at 8.20 or 8.21 log CFU/L, a density largely above that usually encountered in this groundwater. Counts of the newly introduced population were performed over time. In the tested samples, the natural *B. cereus sl* population was below the detection threshold (3.48 log CFU/L).

After inoculation, the newly introduced vegetative cells of *B. cereus* R2SK showed a high constant death rate during the first 10–15 days (Figure 4). After this period, the total *B. cereus* R2SK population reached a low density ranging between the detection threshold and 4 log CFU/L. The level of this surviving *B. cereus* R2SK population did not significantly change over time until 38 days of incubation, after which the density of the viable *B. cereus* R2SK cells decreased below the detection threshold. Thermoresistant *B. cereus* R2SK CFUs were occasionally detected at levels slightly above the detection threshold, indicating that *B. cereus* R2SK cells could sporulate under these conditions or that sporulation may have occurred before inoculation at a level below the detection threshold.

(2) In Groundwater Inoculated with Spores. Groundwater was inoculated with spores of *B. cereus* strain R2SK at 7.67 or 6.19 log CFU/L (a density largely above that usually encountered in this groundwater in natural conditions but a density of only 1 to 3 log higher than the highest density encountered

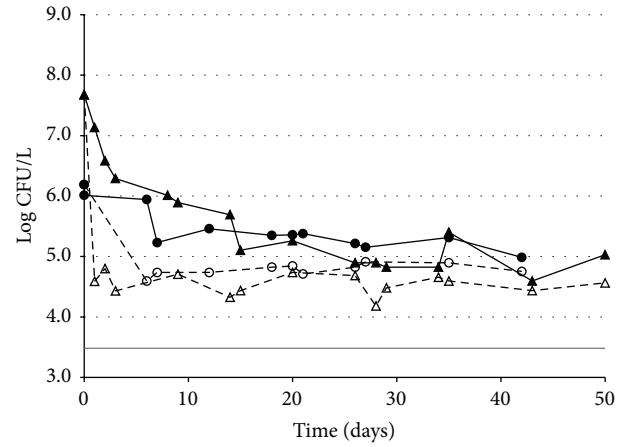

Figure 5: Fate of *B. cereus ss* spores introduced in groundwater. Counts of CFUs and thermoresistant CFUs (i.e., spores) were performed over time. Two independent experiments (triangles, replicate 1; circles, replicate 2) are presented. Full symbols and full lines: *B. cereus ss* total CFU; open symbols and dashed lines: *B. cereus ss* thermoresistant CFU; grey horizontal line: detection threshold (3.48 log CFU/L).

in lysimeter leachates), and counts were performed over time (Figure 5). A rapid decrease in the number of thermoresistant CFUs was observed during the first few days, reaching a steady density of approximately 4.5–5 log CFU/L after less than five days. The number of remaining viable spores seemed extremely stable over time (for up to 40–50 days after inoculation). These results suggest that a proportion of any of the spore population reaching the groundwater environment could persist there for a long time.

The total culturable *B. cereus* R2SK population (i.e., spores plus vegetative cells) also displayed a rapid decrease during the first days after inoculation. However, the decrease was slower than that observed for the counted spores alone. This result indicates that rapid (but incomplete) germination occurred in the groundwater tested. For up to 20 days of incubation, the total *B. cereus* R2SK population reached a density of approximately 5–5.5 log CFU/L, which was at least 1 log CFU/L higher than the density of spores, indicating that a significant proportion of the *B. cereus* R2SK was viable as vegetative cells in this environment. The presence of vegetative cells was confirmed by microscopic observations (data not shown). Then, this total *B. cereus ss* population decreased slightly over time, up to 40–50 days, but was still at a higher density than spores. Under these conditions, the vegetative cells seemed well adapted to this groundwater environment, because these cells were able to survive for at least 50 days.

4. Discussion

Bacillus cereus sl are soil dwelling bacteria, which include foodborne pathogenic strains, that are able to adapt to a wide range of environments. However, little is known concerning the occurrence, fluxes, or fate of *B. cereus sl* bacteria in various aquatic environments (rain, leachates, or groundwater) that are connected to the main reservoir of these bacteria (i.e., soil).

4.1. B. cereus sl Is Present in Rainwater. In recent years, an entire microbial ecosystem has been identified in the atmospheric environment, including rain and fog water [16–18, 48], particularly through the development of large-scale molecular methods (metagenomic approaches). To our knowledge, *B. cereus sl* has not been described as a regular member of this microbial ecosystem, whereas the spore-formers *Bacillus* spp. were observed in the atmosphere [17] and during dust events [49, 50]. We detected *B. cereus sl* in each of the four rain samples collected 2 m above ground level. Thus, *B. cereus sl* bacteria appeared to belong to the normal microbiota of rain. Delort et al. reviewed several studies of microorganisms found in atmospheric water (including rainwater) [51]. Among three major studies, the levels of total bacteria ranged from 6 to 9 log/L (by microscopic cell count); the authors also stated that less than 1% of the collected bacteria could be cultivated on nonselective media [51]. These results are in agreement with the CFU count in the rainwater collected during our study. Similarly, we cannot rule out the possibility that an additional viable but nonculturable *B. cereus sl* population is present in our samples. However, whether *B. cereus sl* occurs in rain drops as free spores or attached to dust particles remains to be investigated. It would be interesting to determine whether *B. cereus sl* found in raindrops originates from soil dust that has been aerosolized and is potentially associated with long-distance dispersal, as suggested by Kellogg and Griffin [49], or directly originates as spores that may have reached the stratosphere with ascending airflows, as already suggested for other bacteria [52].

4.2. Regular Occurrence and Behaviour of B. cereus sl in an Agricultural Soil

4.2.1. B. cereus sl Is Present in Soil as Spores. As hypothesised, we identified *B. cereus sl* strains in the investigated agricultural soil, at a level similar to those levels previously described in other soils [22, 53]. This level seemed constant during two different seasons and at various soil depths. Usually, total bacterial numbers in soil are considered to decrease with depth, because of the decrease in carbon availability [54]. This possibility was confirmed by our microrespirometric data showing a higher active microbial biomass content in the upper layers of the soil investigated. In the soil studied in this research, the natural *B. cereus sl* soil population consisted almost exclusively of dormant spores. Spores do not depend on available organic matter to survive, and this finding may explain why the numbers of *B. cereus sl* did not decrease with depth similar to total bacteria. The data for *B. cereus sl* bacteria in relation to soil depth are scant. A previous study showed that a *B. thuringiensis* population introduced on the surface of a soil by spraying six years before counting significantly decreased at a depth below 10 cm [30]; however, the density of natural *B. cereus sl* isolates at various depths was not investigated.

The observed shift of a *B. cereus ss* population from vegetative cells to spores when introduced into a nonfavourable environment, such as the raw soil used in this study, is in agreement with previous studies [30, 43, 55]. Spores that arose from the newly introduced *B. cereus ss* model population seemed to settle efficiently in the tested soil, with their level remaining highly stable over time, up to ten months. Experimental data concerning the long-term survival of *B. cereus sl* spores are scant. *B. anthracis* was shown to stay viable in a slurry of soil for six months under laboratory-controlled conditions [31], and *B. thuringiensis* was able to survive for at least thirteen years in the field [56].

4.2.2. Lack of Observed B. cereus ss Growth in Soil. Previous studies suggest that *B. cereus sl* spores become active when an excess of easily decomposable organic matter is bioavailable or when the soil presents a high moisture level [57]. In our study, the counts failed to reveal any major increase in the newly introduced *B. cereus ss* model population in this bare agricultural soil; however, this soil was not highly moistened, was not enriched with nutrients, and contained its own microbiota. We also investigated the fate of the introduced *B. cereus ss* population in similar soil samples "cleared" of their own microbiota by treatment with chloroform fumigation, and no change in the level of the newly introduced *B. cereus ss* population was observed (Brillard and Bérard, unpublished data). This finding suggests that a putative competition with the natural microbiota of the tested soil is not responsible for the rapid sporulation of the introduced *B. cereus ss* population.

4.2.3. Determinants Allowing B. cereus sl Multiplication. Because the *B. cereus sl* population in the studied soil was constant over time (at 6-month intervals) and because rainwater

that permits reinoculation of soil contained a lower density of *B. cereus sl* population than percolated water, causing its elimination, other sources of bacteria or conditions allowing *B. cereus sl* multiplication (i.e., "hot spots" of organic matter) must sometimes occur. The term "incubator area" describes the hypothesis of Van Ness, whereby certain soil conditions may favour *B. cereus sl* vegetative growth in "hot spots" of microbial activities, such as the rhizosphere [26]. For instance, it was previously shown that the numbers of *Bacillus* CFUs counted in different rhizosphere samples were 1 log higher than in the control soil without plants [58]. Roots and mycorrhizal exudates represent a major source of dissolved sugars, amino acids, and other organic acids in soil [59], providing highly heterogeneous microenvironments [60] and driving the structure and dynamics of microbial communities [61, 62]. At least 6 free amino acids are able to trigger *B. cereus sl* germination [63] and may be present in such enriched environments. Moreover, alternatives to sporulation were described in studies showing that *B. anthracis* forms biofilms and persists as a vegetative form in a model rhizosphere system [27, 31]. The soil used for *B. cereus ss* inoculation experiments was collected in March 2012, several months after the last cultivated plant (durum wheat harvested in June 2011). This soil was quite poor in organic matter (organic carbon content 13.2 g kg^{-1}) and roots compared with some grassy meadow (36.1 g kg^{-1}) or forest soils, for instance [34], providing an environment less favourable for the multiplication of the *B. cereus ss* population. Additionally, the favourable conditions for *B. cereus ss* growth most likely occur at microspatial and microtemporal scales, which explain the stability and homogeneity of our larger scale *in situ* observations.

More ecological studies are required to clarify the environmental determinants allowing *B. cereus sl* growth in soil with different strains (using isolates originating from this soil, for instance), different soils, and different incubation conditions (e.g., higher humidity, addition of different sources of carbon, etc.), or in association with a rhizosphere or with soil organisms to test the effect of physicochemical conditions and to provide new sources of organic matter for the growth, germination, survival, and sporulation of these populations.

4.3. *B. cereus sl* Can Be Leached Out of Soil and May Reach Groundwater

4.3.1. B. cereus sl Is Present in Leachates. The *B. cereus sl* density in percolated water was up to 3 log higher than that encountered in rainwater, suggesting that *B. cereus sl* CFUs were leached out of soil by water seeping through the ground. Given the size of bacteria (a few microns) and the fact that bacteria are most often adsorbed onto larger soil particles, most of the transport process of bacteria in soil would occur in the largest pore sizes of the soil [64, 65]. The largest pore sizes are also where the preferential flow, bypassing the soil matrix, occurs under natural soil conditions (e.g., [66]). Such preferential flows during high rainfall intensity events have been shown previously in this lysimeter and piezometers of the experimental site [33, 37]. The spore exosporium is believed to play a key part in its attachment to soil substrates and, therefore, is assumed to restrict spore dispersal [24]. In this study, the spores may still be attached to or even encompassed into small soil particles during leaching events. Additional knowledge concerning the *B. cereus sl* attachment and detachment processes [64, 67] should improve our understanding concerning the transport of these bacteria from soil to groundwater.

4.3.2. B. cereus sl May Reach Groundwater. In this experimental field context, the aquifer is located only a few meters below the percolated water collection lysimeter equipment. Thus, our data led us to reasonably hypothesize that *B. cereus sl* can be transferred from soil to groundwater via leachates. We observed *B. cereus sl* in some of our groundwater samples but at an extremely low density. A sandy gravel layer of 2-3 m can be found between soil and groundwater. Pore size exclusion phenomena rather than hydrophobic interactions between spores and soil organic content seem to play an important role in the filtration process in soils [65, 68]. However, an earlier study investigating infiltration processes in the same experimental site suggested that preferential flows observed during high rain events had a quantitative effect on this alluvial aquifer recharge in only isolated cases but could induce local short-term contamination of the groundwater [33].

The groundwater sample that did not display any *B. cereus sl* was sampled in spring 2012 after an extremely dry winter (37 mm between December 2011 and April 2012). The *B. cereus sl* density in water samples and flows observed from the lysimeter leachates were lower during the April 2012 rain event than during 2011 rain events. Garel et al. [33] suggested that the soil capacity to transfer water downwards depends on the rainfall features and on the prior soil moisture conditions because the preferential water flows through the soil depend on the initial water content (and on the saturation of soil macropores) [69]; under wet conditions, the rainfall event of November 2011 (163 mm) may have had an effect on groundwater, whereas in a drier historic context the lower rainfall event of April 2012 (50 mm) may have had less influence on the groundwater. Therefore, *B. cereus sl* occurrence in groundwater could be linked to climatic conditions and seasons (after heavy rain in a wet context, e.g.).

4.3.3. Fate of B. cereus ss in Groundwater. Because *B. cereus sl* may reach groundwater after being leached out of soil, the survival, germination, or even growth could occur in this aquatic environment.

Introduced in groundwater samples as vegetative cells, the model strain of *B. cereus ss* primarily died rapidly; however, a small part remained viable over time. These results suggest that only a minority of the vegetative cells previously grown on rich laboratory media were adapted to survive in a low-nutrient environment such as this groundwater sample. In contrast, a significant part of the *B. cereus ss* population introduced as spores in these groundwater samples survived. Interestingly, germination occurred, and vegetative cells arising from these spore germination events seemed well adapted

to this low-nutrient environment, being able to survive for at least 50 days. Nutritional cues for spore germination may be present as traces in groundwater and could explain the observed *B. cereus sl* germination in such environment. For instance, several free amino acids are known to trigger *B. cereus sl* germination [63]. A different hypothesis would be that the germination is triggered by biological signals emitted by bacterivorous protists inhabiting groundwater. Recent data indicate that, in response to factors excreted by amoebae, *B. cereus sl* can escape ingestion but can also germinate and grow [70, 71].

Because of the rapid decrease in *B. cereus ss* counts observed during our experiments and because of a high detection threshold, groundwater samples were inoculated at high densities, (i.e., spores were inoculated at a density of 1 to 3 log higher than the highest density encountered in lysimeter leachates). Thus, our results only provide an overview of the possible fate of *B. cereus ss* in this environment but should require more investigation to decipher what occurs under more natural conditions. Taken together, these data suggest that a proportion of any spore that reaches the groundwater environment may persist there for a long time as both spores and vegetative cells.

Thus, the occurrence of *B. cereus sl* in groundwater, even at low levels, may not be only temporary. Therefore, risks of the dissemination of *B. cereus sl* via groundwater should be considered in future ecological studies of these bacteria.

4.4. Environmental Isolates Are Diverse. The high diversity of *B. cereus sl* isolates originating from soil, as observed by a fingerprint method, is consistent with previous results showing much diversity among the *B. cereus sl* population in temperate soils [72–75]. The agricultural field area investigated in this study had never been treated by *B. thuringiensis* (as a pest control agent); however, such utilisation may have occurred in the neighbouring agricultural area, and we cannot exclude the possibility of contamination by *B. thuringiensis* strains [76]. However, the high diversity of isolates originating from soil suggests that a major occurrence of the *B. thuringiensis* pest control clonal strain in our soil samples can be ruled out.

No particular group of isolates was identified in our lysimeter percolated samples, suggesting that no particular strains could be leached out of the soil. However, additional investigations should be performed on *B. cereus sl* diversity (in terms of functional and pathogenic traits) in environmental samples linked to transport among the unsaturated zones of the soil for a better risk assessment of ground water contamination.

5. Conclusions

To our knowledge, this study provides a first description of the fate and transport of *B. cereus,* which is a foodborne pathogen, in rain, soil, soil leachate, and groundwater in the context of a field study. Despite using culturable methods that are expected to underestimate the densities of bacterial populations, the presence of *B. cereus sl* was detected in various environmental samples, including rain and groundwater compartments, which have rarely been described. In the investigated soil, the *B. cereus sl* population seemed constant over time; however, after high rain periods, *B. cereus sl* was leached out of soil suggesting that *B. cereus sl* may be transferred to groundwater, where it could persist for a long period.

This descriptive study also opens new fields for future research. This study revealed or confirmed that more ecological studies are required (i) to determine the origin of *B. cereus sl* strains isolated in rain samples, (ii) to clarify the determinants allowing *B. cereus sl* growth in the main reservoir (i.e., soils), (iii) to understand the attachment and detachment processes to/from the soil matrix linked to rain events, (iv) to specify the role of the various ground layers in probable filtration of bacteria before they could reach groundwater, and, finally, (v) to determine the adaptive potential of human pathogenic strains to new environments compared with nonpathogenic ones, given the high diversity of strains observed in environmental samples. The possible fate of *B. cereus sl* in groundwater should however require more investigation to decipher what occurs under more natural conditions (i.e., with lower cell densities).

Depending on the type of food, it was proposed that the contamination by *B. cereus sl* through soil is rarely the main or the only one [4]. Although the question of *B. cereus sl* transfer to foods via diverse routes and at diverse steps of food processing requires more investigation, this study brings new insights about *B. cereus sl* occurrence and behavior in the environment, especially into the water cycle, which may represent yet unstudied routes for food contamination. A proposed model of a possible part of the *B. cereus sl* life cycle is represented in Figure 6. Soil is a reservoir of dormant spores of *B. cereus sl.* When particularly favorable conditions are encountered (presumably at specific spatial and temporal scales), growth may occur so that the pool of *B. cereus sl* is maintained in the soil. Therefore, soil may provide a source for the occasional contamination of rainwater (possibly via air dust) and of groundwater (in which *B. cereus sl* may survive) via leachates and, consequently, could contribute to *B. cereus sl* propagation in distant environments. Thus, rain and groundwater should be considered when studying the *B. cereus sl* life cycle in the environment, particularly in the context of agroecosystems submitted to climatic changes (i.e., heavy rains). Considering these environmental sources of *B. cereus sl* may be helpful for the more accurate assessment of the risk of food poisoning, particularly in ready-to-eat food, by pathogenic strains of *B. cereus sl.*

Abbreviations

B. cereus sl: *Bacillus cereus sensu lato*
B. cereus ss: *Bacillus cereus sensu stricto*
DW: Dry weight
SEM: Standard error of the mean
SIR: Substrate-induced respiration
Spc: Spectinomycin
LB: Luria Bertani
CFU: Colony forming unit

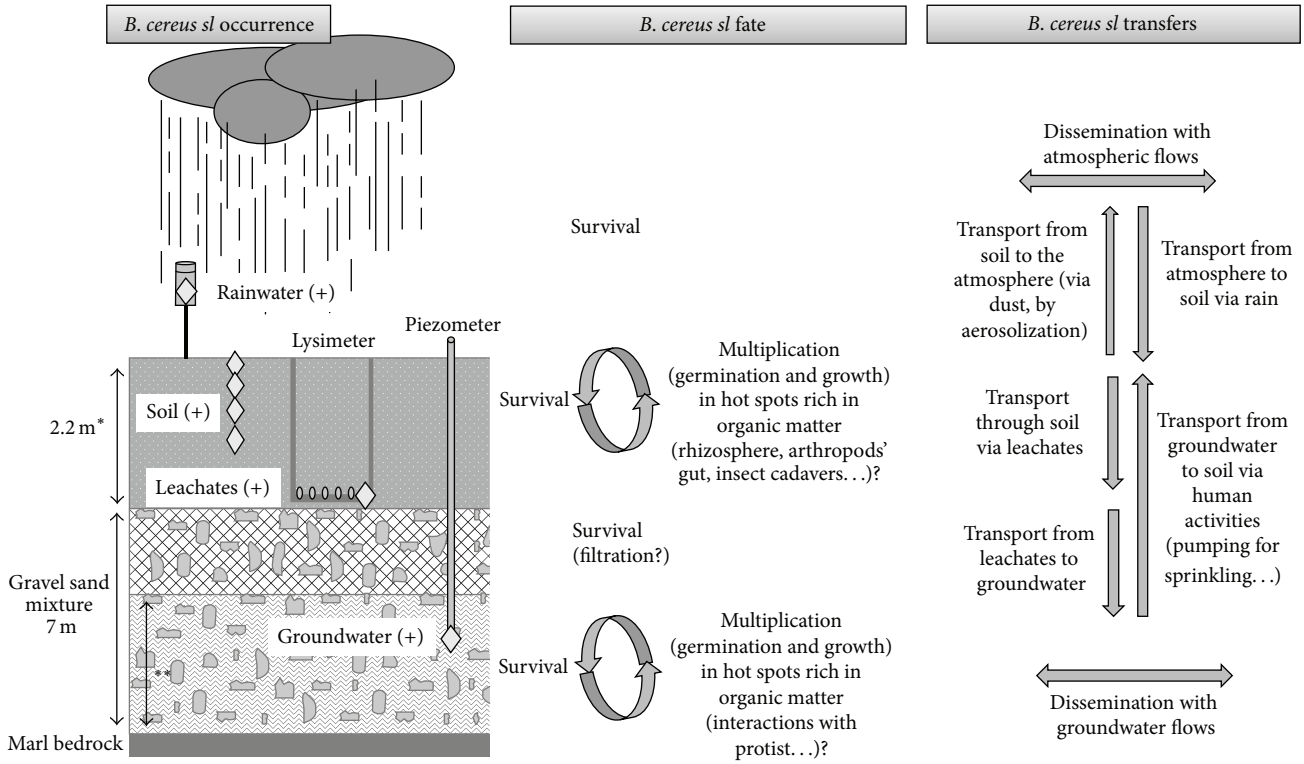

FIGURE 6: Schematic representation of the lysimeter plot and a proposed model of *B. cereus sl* occurrence, fate, and transfers in the agricultural site. The occurrence of *B. cereus sl* was investigated during this study (white diamonds) and observed primarily as spores in the indicated compartments ("+" symbol). *Height of the lysimeter. The soil layer can be slightly deeper or thinner elsewhere on the agricultural plot. **Water table depth ranges from 4 to 5.5 m, depending on the season.

TSA: Trypticase soy agar
MYP: Mannitol-egg yolk-phenol red-polymyxin-agar.

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

Attachment of *Asaia bogorensis* Originating in Fruit-Flavored Water to Packaging Materials

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The objective of this study was to investigate the adhesion of isolated spoilage bacteria to packaging materials used in the food industry. Microorganisms were isolated from commercial fruit-flavored mineral water in plastic bottles with flocks as a visual defect. The Gram-negative rods were identified using the molecular method through the amplification of a partial region of the 16S rRNA gene. Based on the sequence identity (99.6%) between the spoilage organism and a reference strain deposited in GenBank, the spoilage isolate was identified as *Asaia bgorensis*. Experiments on bacterial adhesion were conducted using plates made of glass and polystyrene (packaging materials commonly used in the beverage industry). Cell adhesion ability was determined using luminometry, plate count, and the microscopic method. The strain of *A. bogorensis* was characterized by strong adhesion properties which were dependent on the surface type, with the highest cell adhesion detected on polystyrene.

1. Introduction

Microbial spoilage of fruit-flavored mineral water usually originates during the production process. The raw materials, factory environment, the equipment, and packages, as well as lack of process hygiene, are possible sources of contamination [1]. Spoilage follows from metabolic processes that cause beverages to become undesirable or unacceptable for human consumption, due to changes to their sensory characteristics. Mineral waters with fruit juices or flavors are characterized by high water activity and high levels of vitamins and minerals, which make them good environments for the growth of spoilage microflora [2]. Due to the low pH level of these soft drinks, the predominant spoilage microfloras are acidophilic microorganisms which have developed tolerance towards preservatives used in beverage production. However, new exotic fruit ingredients used in soft drinks can introduce unusual spoilage species with unknown resistance to food preservatives.

The type of packaging used, such as cans and bottles, can also affect the development of spoilage microflora. The material may influence the number and type of cells that grow and adhere to the bottle surface, while the ability of microbial cells to adhere and accumulate on packaging materials can exacerbate contamination of the beverage, reducing its quality and microbiological safety [3, 4]. Packaging materials also vary greatly in terms of oxygen permeability. Glass is still the preferred packaging material for high quality fruit beverages, although the hot-fill/hold/cool process must be applied with care, in order to avoid container breakage. The growth of bacteria is also significantly enhanced by contact with the inner surface of bottles (the so-called bottle effect) [5]. Polystyrene (PS) is one of the plastic materials used most commonly in containers, lids, and bottles. PS is inexpensive, flexible, durable, and chemically resistant [6, 7]. However, the oxygen content in plastic bottles increases with time, whereas glass bottles are impermeable to oxygen [8].

The objective of this study was to identify the spoilage microflora that forms characteristic flocks in commercial bottled fruit-flavored mineral waters and investigate their bacterial adhesion to both glass and polystyrene packaging materials used in the food industry.

2. Materials and Methods

2.1. Isolation of Spoilage Microorganisms. Bacteriological analysis was performed on ten samples of spoiled commercial fruit-flavored mineral water (8.1% sucrose (w/v), 0.05% fruit flavor (w/v), 0.16% citric acid (w/v), 0.02% sodium benzoate (w/v), and 0.02% velcorin (w/v)) from polystyrene bottles. Quantitative examination of the samples was conducted using the pour plate method by inoculating GC agar medium (0.1 mL) with 2% D-glucose (w/v), 0.3% peptone (w/v), 0.3% yeast extract (w/v), and 0.7% $CaCO_3$ (w/v) [9]. Incubation was conducted at 25°C. The characteristic colonies obtained were picked up from the plates, restreaked to ensure purity, and maintained at 20°C on GC agar slants.

2.2. Identification of Spoilage Bacteria. The following standard methods were used for identification: Gram staining, the aminopeptidase test (Bactident Aminopeptidase, Merck), the oxidase test (Bactident Oxidase, Merck), and the catalase test (Bactident Catalase, Merck). Identification was also performed using the PCR technique. For DNA extraction, the strain was cultured on Orange Serum Agar (Merck) for 24 h and the genomic DNA was isolated using a Genomic Mini Kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions.

The 16S rRNA gene was amplified by a polymerase chain reaction (PCR). The reaction was performed in a total volume of 50 μL comprising 24 μL REDTaq ReadyMix DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA), 24 μL PCR grade water, 1 μL of template DNA (50 ng), and 0.4 μL of each primer solution (100 μM). A primer set with the sequences 5′-AGAGTTTGATCCTGGCTCAGAT-3′ and 5′-CGGCTACCTTGTTACGAC-3′ was used [10]. The reaction was carried out in a MJ Mini Thermal Cycler (Bio-Rad, Hercules, CA, USA) with initial denaturation at 94°C for 2 min, followed by 39 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, elongation at 72°C for 3 min, and a final extension step at 72°C for 2 min.

The PCR products were separated using 1% agarose in 0.5 × TBE buffer with ethidium bromide and purified using a Clean Up Mini Kit (A&A Biotechnology, Gdynia, Poland), following the manufacturer's protocol. The nucleotide sequences of the detected bacterial strain were obtained using the BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the reaction products were analyzed using an Applied Biosystems model 3730 Genetic Analyzer (Genomed, Warsaw, Poland). The nucleotide sequences then were compared with 16S rRNA gene sequences of *Asaia* sp. obtained from the National Center for Biotechnology Information (NCBI) using the program BLASTN 2.2.27+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [11]. Multiple alignments of the sequences derived from the reference strain and the identified *Asaia* strains were performed using the Clustal W algorithm. Phylogenetic relationships were inferred using the neighbor-joining method in MEGA5 [12, 13]. No positions containing gaps were considered in the phylogeny analysis. All reconstructions were tested by bootstrapping with 1000 replicates. The evolutionary distances were computed using the maximum composite likelihood method and given in units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. The final dataset comprised a total of 1347 positions.

2.3. Bacterial Cultures. The isolated strain of *Asaia bogorensis* was stored in liquid GC medium (0.3% peptone (w/v), 0.3% yeast extract (w/v), and 0.7% $CaCO_3$ (w/v)) with 2% of D-glucose (w/v) at 4°C [10]. Commercial flavored mineral water with saccharose as a carbon source was used for aerobic cultivation. Due to the presence of thermosensitive ingredients, this medium was sterilized by filtration using a 0.45 μm pore-size Millipore filter. The culture medium (20 mL) was then poured into 25 mL Erlenmeyer flasks, into which sterile carriers were placed vertically in such a way that half of the carrier was immersed in the medium, while the other part remained outside. The amount of inoculum was standardized to obtain a cell concentration in the culture medium approximately equal to 5000–10000 CFU/mL at the start of the experiment. The samples were incubated at 25°C on a laboratory shaker (135 rpm) for 10 days.

2.4. Solid Carriers. Two main types of material were assessed: Star Frost 76 × 26 mm white glass slides (G) (Knittel Glass, Germany), used as the reference material, and rectangular discs of polystyrene (PS) 76 × 26 mm (Paccor Packaging Poland, Skierniewice), a material certified by the Polish National Institute of Public Health and approved for contact with food.

2.5. Determination of Contact Angle and Surface Tension. The contact angle values for the two different solvents, dimethylformamide (DMF) and water, were determined using a ramé-hart NRL goniometer (ramé-hart instrument co., Succasunna, NJ, USA) equipped with a JVC KYF-70B camera (JVC, Yokohama, Japan). The dynamic contact angle was calculated using DROPimage software (ramé-hart instrument co., Succasunna, NJ, USA), as the average of 15 measurements. The total surface tension was calculated from the values of the contact angles of solvents with different polarities (Owens-Wendt method) [10].

2.6. Assessment of Bacterial Adhesion. The analysis of adhesion to the carriers was performed using luminometry, the plate count method, and microscopic observations. For the luminometric tests, the carrier plate was removed from the culture medium, rinsed with sterile distilled water and swabbed using free ATP sampling pens (Merck, Germany). Measurements were reported in relative light units (RLU) using a HY-LiTE 2 luminometer (Merck) [10]. The colony count method was used to determine the number of viable bacterial cells, both on the tested surface and in the culture medium. The carrier plate was removed from the culture medium, rinsed with sterile distilled water, and swabbed using sterile swabs for surface testing. The bacterial suspensions were vortexed vigorously in 0.85% saline solution with 0.1% Tween 80 for 5 min and transferred onto GC agar. After incubation (25°C, 96 h), the characteristic pink colonies of

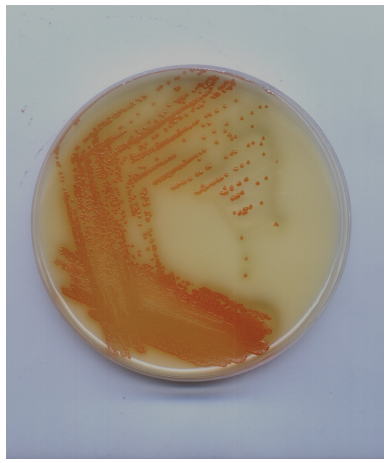


Figure 1: Growth of the dominant morphotype of AAB on GC agar medium.

Asaia spp. were counted and the number of attached bacterial cells per square centimetre of the carrier was determined. The relative adhesion coefficient (A) was then calculated: the total number of adhered cells was divided by the total number of planktonic cells in the given sample. The coefficient A was expressed as a percentage value [14].

In the microscopic studies, *Asaia* spp. cells were stained with basic fuchsin (0.5%). Bacterial cells on the carrier were observed using an OLYMPUS type BX41 light with DP72 digital camera. The total cell adhesion area in the observation field was evaluated using UTHSCA Image Tool software (http://compdent.uthscsa.edu/dig/itdesc.html).

Mean values were calculated from the data obtained from the three independent experiments. Comparisons between the mean values were performed using the one-way ANOVA test (STATISTICA 10, StatSoft, Poland).

3. Results and Discussion

3.1. Isolation and Identification of Spoilage Microorganisms. The samples of spoiled fruit-flavored mineral water (pH = 3.3) had characteristic, visually observable flocks. Incubations demonstrated the presence of mixed cultures consisting of two main morphotypes, from which single colonies were isolated. One of these morphotypes was the yeast *Rhodotorula* spp. However, the second was dominant and very characteristic—the bacteria grew best at 25°C on agar medium with glucose, peptone, and $CaCO_3$, forming small, pale, smooth, orange-pink colonies with clear zones after 72 h of incubation (Figure 1). Cells of this morphotype were Gram negative, catalase positive, oxidase negative, and rod-shaped, measuring 0.5–1.0 × 0.8–2.0 mm. The phenotypic characteristic of the isolate obtained from the beverage was consistent with that described previously for acetic acid bacteria of the genus *Asaia*.

AAB are known for the high frequency of their spontaneous mutations [15]. Therefore, polyphasic procedures should be followed based on both classical phenotypic tests and genotypic methods. It has been shown that using a single feature as the sole diagnostic criterion for AAB species definition is unreliable. In our study, API identification tests were also unable to identify the AAB with high confidence (unacceptable profiles), so identification was additionally based on the 16S rRNA gene sequence. Direct sequencing of the 16S rRNA gene identified the cells as *A. bogorensis* (99.6% identity with *Asaia bogorensis* NBRC 103528). The phylogenetic relationships determined for the 16S rRNA gene sequences of 8 strains belonging to *Asaia* spp. (*A. bogorensis*, *A. siamensis*, *A. krungthepensis*, *A. lannensis*, *A. spathodea*, *A. astilbis*, *A. platycodi*, and *A. prunellae*) and 2 strains of *Gluconobacter oxydans* and *Acetobacter aceti* are shown in Figure 2. The 16S rRNA gene sequence determined for *Asaia bogorensis* strain FFMW was deposited in the GenBank database with accession number KC756841.

Table 1: The surface free energy (SFE) of solid surfaces [mJ/m^2].

Surface	Total SFE	Dispersive contribution of SFE	Polar contribution of SFE
Glass	54.2 ± 0.4	7.9 ± 0.1	44.3 ± 0.3
Polystyrene	40.6 ± 0.3	34.3 ± 0.2	6.3 ± 0.1

Asaia bogorensis strains were first isolated from orchid tree flowers (*Bauhinia purpurea*) and plumbago flowers (*Plumbago auriculata*), as well as from fermented glutinous rice by Yamada et al. [16]. The hydrophilic cells are able to synthesize extracellular cellulose in a solid pellicle at the air-liquid interface of static culture media [17, 18]. This ability may contribute to the formation of biofilms on many types of surface commonly used in food processing [4]. The results obtained from our experiments suggest that *Asaia bogorensis* is able to grow in fruit-flavored mineral water despite the combination of low pH and chemical preservatives (sodium benzoate, citric acid, and dimethyl dicarbonate, velcorin). This property has also been noted in studies conducted by Horsáková et al. [3]. *A. bogorensis* does not appear to present a significant risk to human health, although some strains may be opportunistic pathogens in patients with reduced immunity [19–21].

3.2. Surface Tension of Packaging Materials. Surface tension is one of the most important physicochemical properties of any solid surface and is correlated with resistance to biofilm formation. A general relationship between surface tension and the relative amount of adhesion was found to form a "Baier curve," with the zone of minimal adhesion in the region between 20 and 30 mJ/m^2. High bioadhesion occurred when this parameter exceeded 30 mJ/m^2 [22]. The values for tested glass and polystyrene were different (their measured surface energies varied from 40 to 54 mJ/m^2) but in both cases they exceeded the critical value of 30 mJ/m^2. The polystyrene exhibited lower surface tension than the glass surface (Table 1). This fact might indicate that PS is more adhesive. However, the adhesion properties of tested surfaces

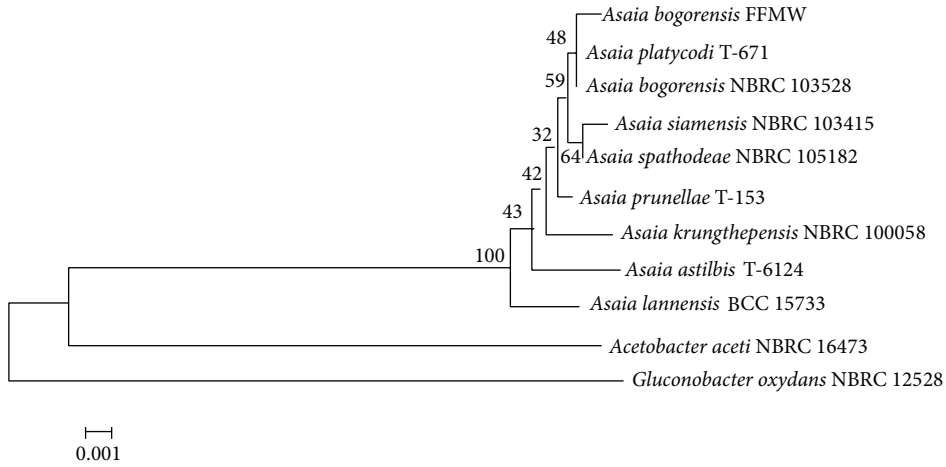


Figure 2: The phylogenetic tree constructed on the basis of 16S rRNA gene sequences of *Asaia* reference strains, *Gluconobacter oxydans* and *Acetobacter aceti*. The tree was constructed using the neighbor-joining method and tested by bootstrapping (1000 replicates). Only branches with 50% and above support from bootstrapping were shown.

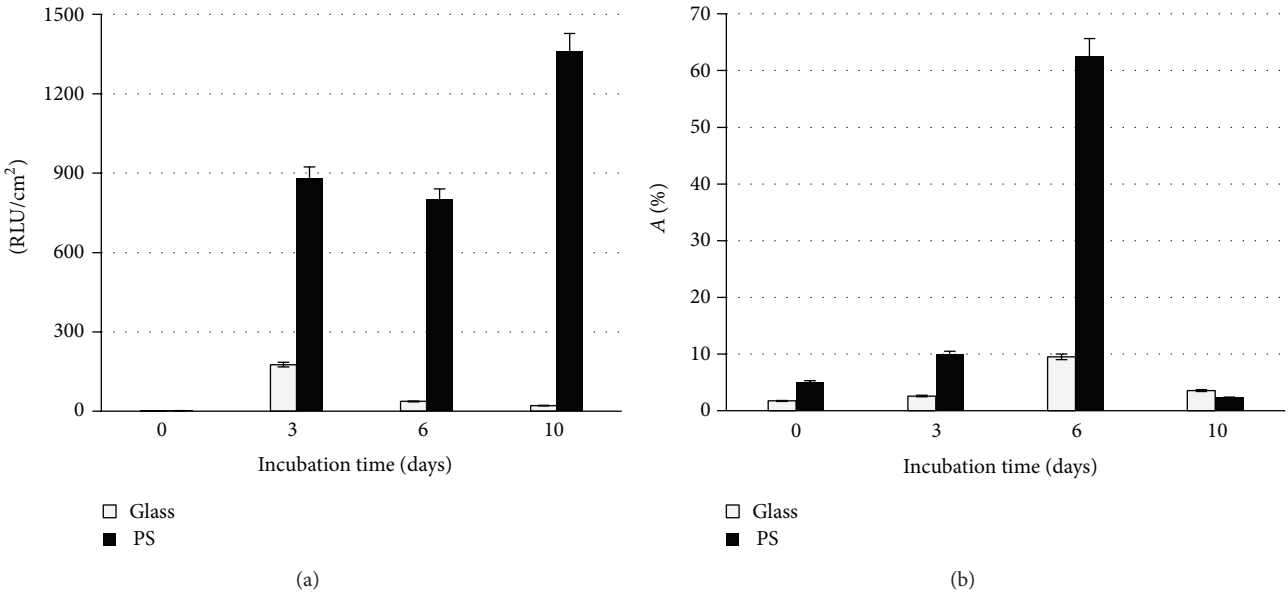
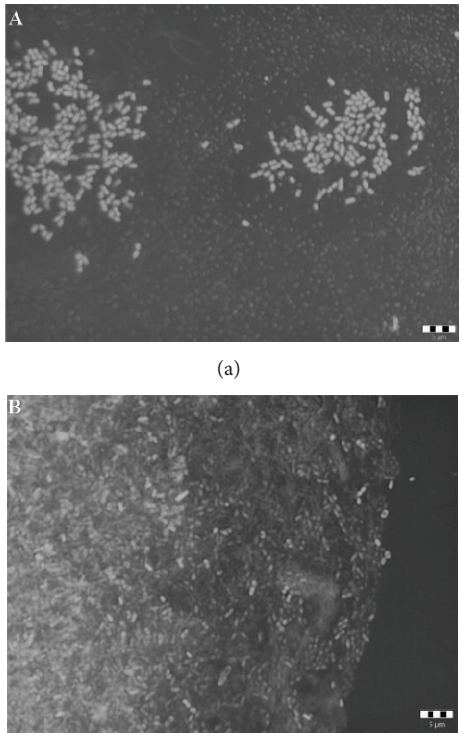


Figure 3: Adhesion of *A. borogensis* in fruit-flavored mineral water: (a) adhesion reported in relative light units per cm^2 and (b) relative adhesion coefficient (A) expressed in %.

can become modified rapidly by immersion in water and by the adsorption of conditioning films [23]. In our study, the tested surfaces were in contact with fruit-flavored mineral water containing organic matter for 10 days, which may have influenced the adhesive events associated with the attachment of bacterial cells.

3.3. Attachment of Asaia bogorensis to Packaging Materials. Biofilm formation may be a survival strategy for starved bacteria, and it has been confirmed that increased cell adhesion is often correlated with nutrient limitation [23]. Therefore, our adhesion studies used commercial flavored mineral water that was poor in nutrients. The intensity of biofilm formation on two packaging materials, glass and polystyrene, was assessed by luminometric measurement and expressed in relative light units (RLU). Figure 3 presents the results of luminescence (RLU/cm^2) and the relative adhesion coefficient A (%) for all tested surfaces. Biofouling during incubation had a dynamic and changeable character. The number of attached *A. bogorensis* cells was significantly higher with polystyrene. After 10-day incubation, the levels of adhesion to glass and PS in the flavoured water were 22 and 1360 RLU/cm^2, respectively. The relative adhesion coefficient for polystyrene was also several times higher in comparison



(a)

(b)

Figure 4: Attachment of *A. bogorensis* cells to (a) glass and (b) polystyrene. Bars represent 5 μm.

to the glass surface. Similar results had been obtained by Kregiel [24] with the *Asaia lannensis* strain and polyethylene material.

In the experiments we also used qualitative analysis of adherent cells based on light microscopy. Figure 4 shows images of glass and polystyrene surfaces stained with fuchsin. Irregular cell adhesion with an extracellular substance was detected on the PS material, resulting in surface coverage ranging from approximately 30% to 50% of the total area. Therefore, glass proved to be more resistant as a packaging material to *Asaia bogorensis* adhesion.

4. Conclusion

This study has identified the presence of *Asaia bogorensis* in samples of defective strawberry-flavored bottled mineral water. The low pH level and addition of chemical preservatives in these products did not prevent the growth of bacterial cells. Adhesion and biofilm formation on the bottle materials were shown to exacerbate contamination by *A. bogorensis*, reducing the quality and microbiological safety of the beverage products. Attachment of *A. bogorensis* cells decreased as the polar surface tension of the substrate increased. Using glass as packaging material, with a high polar contribution of surface tension, allows for a significant reduction of *Asaia* spp. adhesion and may contribute to improving the microbiological stability of fruit-flavored mineral waters.

Conflict of Interests

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

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