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Detection of presumptive *Bacillus cereus* in the Irish dairy farm environment

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Abstract

The objective of the study was to isolate potential *Bacillus cereus* sensu lato (*B. cereus s.l.*) from a range of farm environments. Samples of tap water, milking equipment rinse water, milk sediment filter, grass, soil and bulk tank milk were collected from 63 farms. In addition, milk liners were swabbed at the start and the end of milking, and swabs were taken from cows' teats prior to milking. The samples were plated on mannitol egg yolk polymyxin agar (MYP) and presumptive *B. cereus s.l.* colonies were isolated and stored in nutrient broth with 20% glycerol and frozen at –80 °C. These isolates were then plated on chromogenic medium (BACARA) and colonies identified as presumptive *B. cereus s.l.* on this medium were subjected to 16S ribosomal RNA (rRNA) sequencing. Of the 507 isolates presumed to be *B. cereus s.l.* on the basis of growth on MYP, only 177 showed growth typical of *B. cereus s.l.* on BACARA agar. The use of 16S rRNA sequencing to identify isolates that grew on BACARA confirmed that the majority of isolates belonged to *B. cereus s.l.* A total of 81 of the 98 isolates sequenced were tentatively identified as presumptive *B. cereus s.l.* Pulsed-field gel electrophoresis was carried out on milk and soil isolates from seven farms that were identified as having presumptive *B. cereus s.l.* No pulsotype was shared by isolates from soil and milk on the same farm. Presumptive *B. cereus s.l.* was widely distributed within the dairy farm environment.

Keywords

B. cereus • BACARA • dairy environment • MYP agar

Introduction

Milk and products derived from the milk of dairy cows can harbour a variety of microorganisms that can be important sources of food-borne pathogens. Commercial pasteurisation of milk has been used to eliminate all non-spore-forming bacteria in milk that can cause human disease (Boor and Murphy, 2002). Consequently, the incidence of milk-borne illness is rare, and those reported are mostly due to the consumption of non-pasteurised dairy products or products that have been subjected to post-pasteurisation contamination (Gillespie *et al*., 2003; Langer *et al*., 2012). Unfortunately, pasteurisation will not destroy all food-borne pathogens. *Bacillus cereus sensu lato* (*B. cereus s.l.*) can survive pasteurisation and member species have been successfully isolated from a variety of dairy products (te Giffel *et al*., 1996; Haughton *et al*., 2010). Although illness associated with the consumption of food contaminated with *B. cereus* is relatively rare in adults, their presence in infant milk formula (IMF) has been reported (Becker *et al*., 1994; Johnson *et al*., 2005) and is, therefore, a cause for concern. In one study, of 92 samples of IMF collected from 17 European countries (including Ireland), 54% were positive for *B. cereus* (Becker *et al*., 1994). The number of *B. cereus* organisms recovered was regarded as being too low to cause illness (Becker *et al*., 1994; Haughton *et al*., 2010). However, when contaminated IMF samples were stored under unfavourable conditions after reconstitution (>25 °C for 14 h), it was found that *B. cereus* numbers reached potentially harmful levels (>10^3 colony-forming units [cfu]/g powder; Haughton *et al*., 2010). To protect the health of infants, the European Commission (EC 1441/2007) has set a threshold for presumptive *B. cereus* in dried infant formulae intended for infants <6 months of age. To be satisfactory, five samples of infant formula from a batch are analysed (reference method: International Organization for Standardization [ISO] 7932), and four samples must have a bacterial load of *B. cereus s.l.* <50 cfu/g, while the remaining sample can have bacterial load between 50 and 500 cfu/g. Due to the vulnerability of the consumers of IMF and the large market share that the Irish dairy industry has in the global export market of IMF (International Dairy Federation [IDF], 2013), the production of IMF with minimal contamination of *B. cereus s.l.* is of critical importance to the Irish dairy industry.

The number of *B. cereus s.l.* in pasteurised dairy products is dependent on the initial population in the raw milk. Therefore,

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it is important to minimise transmission of *B. cereus s.l.* into bulk tank milk (BTM) at the farm level. *B. cereus s.l.* is ubiquitous in nature, and spores of the pathogen have been isolated from dairy farm sources, including rinse water from the milking machine, used bedding (Magnusson *et al*., 2007), teats contaminated with soil, dirty alleys (Christiansson *et al*., 1999), faeces and silage (Vissers *et al*., 2007). When cows are kept indoors, spores in used bedding are a major source of contamination of BTM via contaminated teat and udder surfaces (Magnusson *et al*., 2007). However, when cows are grazing outdoors, contamination of teats with soil is the main route of contamination of BTM (Christiansson *et al*., 1999).

Due to the similarity between *B. cereus sensu stricto* (*B. cereus s.s.*) and some *B. cereus s.l.* species, including *B. thuringiensis*, *B. weihenstephanensis* and *B. anthracis*, it is difficult to distinguish between these species using selective agar (US Food and Drug Administration [FDA], 2012). The 16S ribosomal RNA (rRNA) gene is highly conserved (99% identity) among certain species of the group (Priest *et al*., 1994). However, some species such as *B. mycoides* and *B. pseudomycoides* are distinct in their colony morphology (rhizoidal growth) and, therefore, it is possible to distinguish them from *B. cereus s.s.* using quantitative real-time polymerase chain reaction (PCR) (Oliwa-Stasiak *et al*., 2011). The dairy industry in Ireland routinely monitors milk for *B. cereus s.l.* in order to select high-quality milk for IMF manufacture.

The isolation of presumptive *B. cereus s.l.* in the dairy farm environment is yet to be carried out in Ireland. The objective of this study was to identify the potential presence of *B. cereus s.l.* in BTM and in a variety of farm samples using traditional culture and molecular techniques.

Materials and methods

Sample collection

A total of 754 samples (milk, swabs, water, milk filters, soil and grass) were collected from 63 commercial farms located within a 24km radius of the Animal and Grassland Research and Innovation Centre, Teagasc, Fermoy, which were visited at milking time between 4th July and 31st August 2012.

BTM samples (25 mL) were collected from each farm (n = 5) over a 2-wk period prior to the on-farm visit and were taken just before milk was collected by the milk processor (48-h intervals). All these milk samples were analysed for the presence of *B. cereus s.l.* Samples were taken aseptically from the top of the tank after agitation. All the samples were immediately placed on ice and analysed within 24 h of collection.

During the on-farm visit, a variety of farm samples were collected, including soil and grass samples collected from the paddock that the cows had just grazed. Soil samples (100–200 g) were collected using a soil core sampler. Grass samples were collected using a battery-operated handheld clippers (Gardena, Hans-Lorenser-STR 40, 89079, ULM, Germany) to avoid soil contamination. Both the soil core sampler and grass clippers were cleaned with disinfectant wipes between samplings of each farm. A swab sample (swabs were moistened with 0.001% bacteriological peptone [Oxoid] before sample collection) was taken from the inside of one liner within each milking cluster (up to a maximum of 10 clusters) before the start of milking and after the entire herd was milked. Swabs taken from the liners (n = 10) before milking were pooled to form one composite sample. Likewise, a composite sample was formed from the swabs taken from the liners after milking, resulting in two liner-swab samples per farm. Similarly, one swab sample was taken from one teat from up to a maximum of 20 randomly selected cows within the herd before clusters were applied. Again, the teat swabs were pooled to form one teat swab sample per farm. Briefly, each swab was placed in a microfuge tube containing sterile peptone solution. The tubes from each source were vortexed for 1 min, and the solutions were pooled. After milking, the milk filter was collected. A sample of water used for rinsing the milking equipment was collected either directly before or after milking (depending on the washing routine in place on the farm). Additionally, a sample of tap water from the dairy was collected. Soil and grass samples were frozen at –20 °C before analysis. All other samples were analysed within 48 h of collection.

Microbiological analysis

The milk filter from each farm was cut into small squares, weighed and mixed with twice as much weight of sterile peptone and then homogenised using a stomacher for 2 min. Similarly, 25 g of both soil and grass from each farm were added to individual 225 mL volumes of sterile peptone solution (0.002%) and then homogenised in a stomacher. All samples were serially diluted and 1 mL of each sample was surface-plated on two plates (140 mm diameter) of mannitol egg yolk polymyxin agar (MYP) (Merck, Darmstadt, Germany). The plates were incubated at 32 °C for 48 h. Typical colonies of *B. cereus s.l.* (pink colonies surrounded by a zone of precipitation) were counted and further tested to detect the presence of b haemolysis on blood agar plates (horse blood agar, 7% concentration, base No. 2; Oxoid, Basingstoke, UK) following incubation for 24 h at 32 °C. Colonies identified as presumptive *B. cereus s.l.* were purified on brain heart infusion (BHI) agar and frozen at –80 °C in nutrient broth with 20% glycerol for further analysis. Those that presented as typical colonies but were non-haemolytic were stored as a precautionary measure. Additionally, when samples presented with different colony morphology on BHI

agar, each colony type was stored as an individual isolate. Thus, it was possible to have multiple isolates stored for some samples.

Because MYP agar is not very selective for the growth of *B. cereus s.l.* in the presence of competitive bacteria (Tallent *et al*., 2012), all frozen isolates were subsequently surface-plated on BACARA agar (Biomerieux, Hampshire, UK) and incubated at 32 °C for 24 h for further confirmation of the presence of presumptive *B. cereus s.l.* Orange colonies with an opaque halo were manually counted. Before colonies were frozen, they were tested for catalase production using 5 mL of an overnight culture mixed with 3% hydrogen peroxide (Sigma-Aldrich Ireland Ltd., Arklow, County Wicklow, Ireland) on a microscope slide; bubble production was indicative of a positive catalase test. Catalase-positive isolates were also viewed on an Olympus microscope (Olympus BX-51) under the 100× oil Iris objective lens. Images were captured with a DP50 camera (Olympus Co., Toyko, Japan).

Molecular speciation of isolates

Isolates identified as presumptive *B. cereus s.l.* on BACARA agar were analysed using 16S rRNA sequencing. The presumptive *B. cereus s.l.* isolates from BACARA plates were chosen, and DNA was extracted from 10 mL of an overnight culture using the Genelute DNA extraction kit (Sigma-Aldrich), according to manufacturer's instructions. The 16S rRNA was amplified from the genomic DNA (gDNA) of each strain using the eubacterial cytochrome c oxidase subunit I (CO1) and CO2 universal primers under conditions outlined previously (Simpson *et al*., 2003), and the PCR products were sequenced by conventional Sanger sequencing (Beckman-Coulter Genomics). Sequences were analysed and assembled using Lasergene V8 software. The presumptive species of each isolate was determined by nucleotide alignments with sequences of *Bacillus* species deposited in the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) database (performed in October 2015) (http://blast.ncbi.nlm.nih.gov).

Pulsed-field gel electrophoresis (PFGE)

Milk and soil isolates that were isolated simultaneously from several farms and were identified as presumptive *B. cereus s.l.* using 16S rRNA analysis were analysed using PFGE to determine strain similarity. The PFGE analysis was performed with the restriction enzymes *Not*I and *Sbf*I (New England Biolabs, Ireland), in two separate experiments, as described by Drean and Fox (2015). Isolate similarity dendrograms were generated using Bionumerics version 7.5 software (Applied Maths, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimisation settings of 1%, as previously described (Fox *et al*., 2012).

Results and discussion

In total, 754 samples from a variety of farm environments across 63 farms were collected (Table 1). Some samples were negative for *B. cereus,* while in other instances, multiple isolates, reflecting different colony morphology on MYP agar, were retained per sample (Table 1). On average, five BTM samples were taken per farm prior to the on-farm visit. Using MYP agar, 507 isolates from milk, swab, rinse water, tap water, soil and grass were identified as presumptive *B. cereus s.l.* Presumptive *B. cereus s.l.* isolates were found among all sample sources, but the majority of the isolates stored were from milk samples (Table 2). The use of MYP agar and the confirmation of suspect colonies by the presence of b-haemolysis is currently used by at least one milk processor for the detection of most *B. cereus s.l.* (*B.*

Table 1. Sources of samples collected, plated on MYP and stored as isolates

Sample source	Total number of samples collected	Total number of negative samples	Total number of isolates obtained from colonies that grew on MYP agar	Total number of farms with at least one positive isolate
Milk[1]	323	120	193	50
Teat swabs	63	11	75	52
Liner swabs – before milking	49	29	26	20
Liner swabs – after milking	63	25	45	38
Milk filter	45	16	44	29
Tap water	59	35	31	24
Final rinse water	33	24	10	9
Soil	57	11	46	46
Grass	62	26	36	36

[1]Milk samples were taken on the day of the farm visit and days prior to the visit.
MYP = mannitol egg yolk polymyxin agar.

Table 2. The number of potential *Bacillus* spp.-positive isolates identified from dairy farm environments as presumptive *B. cereus s.l.* using MYP, BACARA agar and 16S rRNA sequencing

Detection1	Milk	Teat swabs	Liner swabs – before milking	Liner swabs – after milking	Milk filter	Tap water	Final rinse water	Soil	Grass	Total
MYP	193	75	26	45	44	31	10	46	36	507
BACARA	46	22	7	13	10	7	5	39	28	177
16S rRNA	16	10	4	6	2	4	5	30	4	81

[1]Only samples (n = 507) that were identified as presumptive *Bacillus cereus sensu latos* on MYP agar were surface-plated on BACARA agar. Further confirmatory tests (catalase test, microscope observation) were carried out on samples that were identified as presumptive *B. cereus* on BACARA agar. Only isolates that were catalase positive and appeared as rods under the microscope were subjected to 16S rRNA sequencing (n = 98).
MYP = mannitol egg yolk polymyxin agar.

antracis is non-haemolytic) in raw milk. On MYP agar, *B. cereus s.l.* colonies are tentatively identified on the basis of the production of lecithinase and inability to ferment mannitol (FDA, 2012). Several studies (van Netten and Kramer, 1992; Peng *et al*., 2001; Tallent *et al*., 2012) have highlighted a number of problematic issues associated with MYP agar, including the occurrence of false negatives due to the absence of characteristic colony morphology and production of lecithinase. Additionally, characteristic growth may be masked by the presence of background flora that ferment mannitol and produce lecithinase. Even in the absence of background flora, overlapping precipitation zones and the propensity of *B. cereus s.l.* colonies to coalesce hinders accurate enumeration.

Given that milk supplied for IMF manufacture in Ireland is selected based on its vegetative presumptive *B. cereus s.l.* count, it was decided that samples obtained on farm would not be pasteurised prior to analysis. This approach is different from that of other studies (Slaughis *et al*., 1997; Christiansson *et al*., 1999), in which samples were pasteurised (63 °C for 30 min) prior to plating. The heat treatment of samples would likely reduce levels of members of the background microbiota capable of growing on MYP agar and allow for easier identification of presumptive *B. cereus s.l.* colonies. In this study, the abundant growth of competitive bacteria present in samples interfered with the growth, detection and colony enumeration of *B. cereus s.l.* Due to the abundant growth of mannitol-fermenting organisms on MYP agar in this study, it is likely that many *B. cereus s.l.* colonies were not identified, resulting in false-negative results. On the other hand, had the samples been pasteurised prior to analysis, the concentration of vegetative cells in the samples would have been reduced, making it more difficult to detect. Plating unpasteurised samples ensured maximum recovery of *B. cereus s.l.* from the samples.

A study by Tallent *et al*. (2012) compared MYP and BACARA agars for the isolation of *B. cereus s.l.* in contaminated food samples and found BACARA to be more selective and differential than MYP agar. Thus, it was decided that isolates identified as presumptive *B. cereus s.l.* on MYP should be restreaked on BACARA agar. When these isolates were surface-plated on BACARA agar, only 35% of isolates grew with typical, or almost typical, colony morphology (Table 2). Given that the majority (81 isolates out of a total of 98) of isolates that grew on BACARA agar were tentatively identified as species belonging to the *B. cereus* group using 16S rRNA sequencing, it can be implied that the bacteria that grew on MYP but not on BACARA were unlikely to belong to *B. cereus s.l*. Findings from other studies suggest that MYP is less selective for the identification of *B. cereus s.l.* in BTM than BACARA (Tallent *et al*., 2012).

It is difficult to distinguish most *B. cereus s.l. species* using MYP or BACARA agar due to similar colony morphology (FDA, 2012). Similarly, 16S rRNA gene sequencing is not sufficient to differentiate all the specific *Bacillus* species within the *Bacillus* group (Ash *et al*., 1991). In this study, microscopy was used to differentiate cocci and rods, and in each instance where rods were detected, these samples were taken forward for DNA extraction. The presence of other species was due to a low level of contamination in the samples from the soil, which may have been selected with the *Bacillus* isolates during culturing. BLAST analysis did indicate that the majority (81 isolates from a total of 98) of isolates did belong to the *B. cereus s.l.* (the best BLAST hits were to *B. cereus, B. thuringienisis* and *B. mycoides*).

In addition, the best BLAST hit of the other 17 isolates that grew on BACARA identified the isolates as species such as *Enterococcus faecalis, Lactococcus lactis, Carnobacterium maltaromaticum* and *Enterococcus faecium*. This could be as a result of the presence of false positives on BACARA agar, but, more likely, this reflects the presence of contaminated samples. Indeed, Tallent *et al*. (2012) previously isolated *Enterococcus* species on BACARA agar, which grew as distinct pink pinpoint colonies. These colonies are likely to be masked by larger colonies on BACARA agar, leading to their accidental isolation.

Figure 1A illustrates the typical colony morphology of presumptive *B. cereus s.l.* colonies on BACARA agar, while Figure 1B illustrates the colony morphology exhibited by the majority of soil isolates in this study. This rhizoidal growth pattern shown in Figure 1B is typical of *B. mycoides* species (FDA, 2012). However, Tallent *et al*. (2012) reported that *B. mycoides* growth was inhibited on BACARA agar. Though the use of 16S rRNA sequencing is not discriminatory between *B. cereus s.s.,* the highest BLAST hit of the soil isolates that grew on BACARA by 16S rRNA sequencing were tentatively identified as *B. cereus or B. mycoides/pseudomycoides* species. These findings raise the possibility that *B. mycoides* may be able to grow on BACARA agar. However, further analysis would be required to fully determine this.

Due to the lack of presumptive *B. cereus s.l.* -positive isolates from each sample source on each farm, it was not possible to investigate whether specific strains of *B. cereus s.l.* persisted throughout the milk production chain. However, seven farms presented with both milk and soil isolates that were tentatively identified as members of the *B. cereus s.l.* group using 16S rRNA sequencing. Consequently, it was decided to carry out PFGE on these isolates to determine whether strains similar to those from the soil were present in BTM. The PFGE analysis of the soil and milk isolates indicated that no similar pulsotypes were identified in both soil and milk samples from the same farm (Figure 2). However four milk sample isolates – 290, 399, 403 and 448 – appear to have a similar PFGE pulsotype. In addition, the BLAST analysis for these isolates indicated that they were *B. cereus* OkF01 (99%). Two other milk sample isolates – 416 and 442 – also had a similar PFGE profile. However, these two isolates were identified by BLAST as *Bacillus* sp. cp64 (99%) and *Bacillus* sp. XT-24 (99%), respectively. These variations in BLAST could, however, be due to the limitations of the use of 16S rRNA in differentiation of *Bacillus* isolates. All the milk isolates were obtained from different farms, and there is no apparent connection between these farms to cause contamination with similar strains. However, given the close proximity of all seven farms, the presence of similar strains in the BTM of each farm is not surprising. Indeed, it is possible that the similar strains found in the milk samples are widespread throughout the dairy farm environments of each farm sampled in this study. However, substantially more sampling would be required on each farm to establish the significance of shared pulsotypes between milk and the farm environment.

Although PFGE patterns can be used to differentiate between strains of *B. cereus s.l.,* the frequent presence of plasmids and other extra-chromosomal elements in the *B. cereus s.l.* can cause strains that appear quite similar on PFGE to behave very differently; so using PFGE to compare strains can have limitations (Carlson *et al*., 1994; Ivanova *et al*., 2003).

Conclusions

In this study, two agars (MYP and BACARA) were used to identify *B. cereus s.l.* from BTM and farm samples. The difference in the number of *B. cereus s.s.* organisms detected using BACARA agar compared to MYP agar may be accounted for if all samples were plated in tandem on both media and all isolates sequenced. The use of 16S rRNA sequencing to identify isolates that grew on BACARA

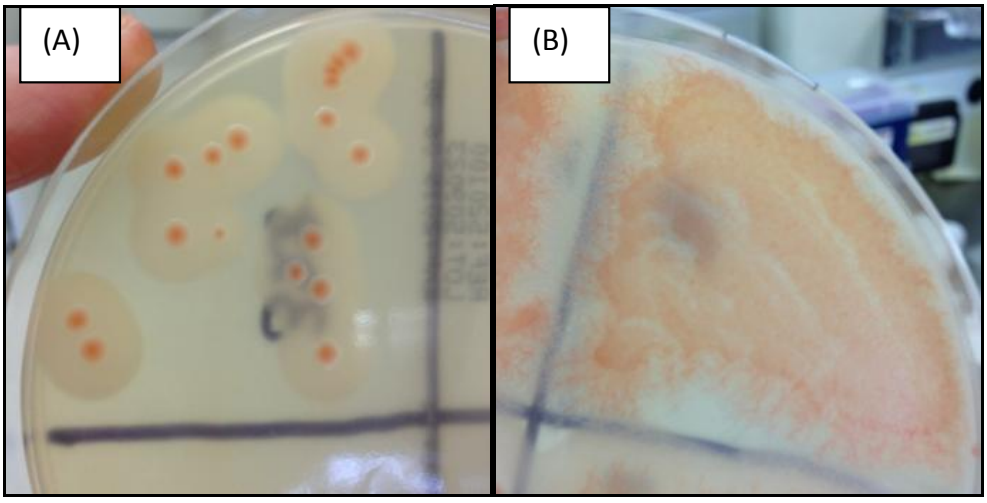

Figure 1. (A) Typical colony morphology of *Bacillus cereus sensu lato* on BACARA agar. (B) Colony morphology of soil samples grown on BACARA agar exhibiting distinct rhizoidal growth.

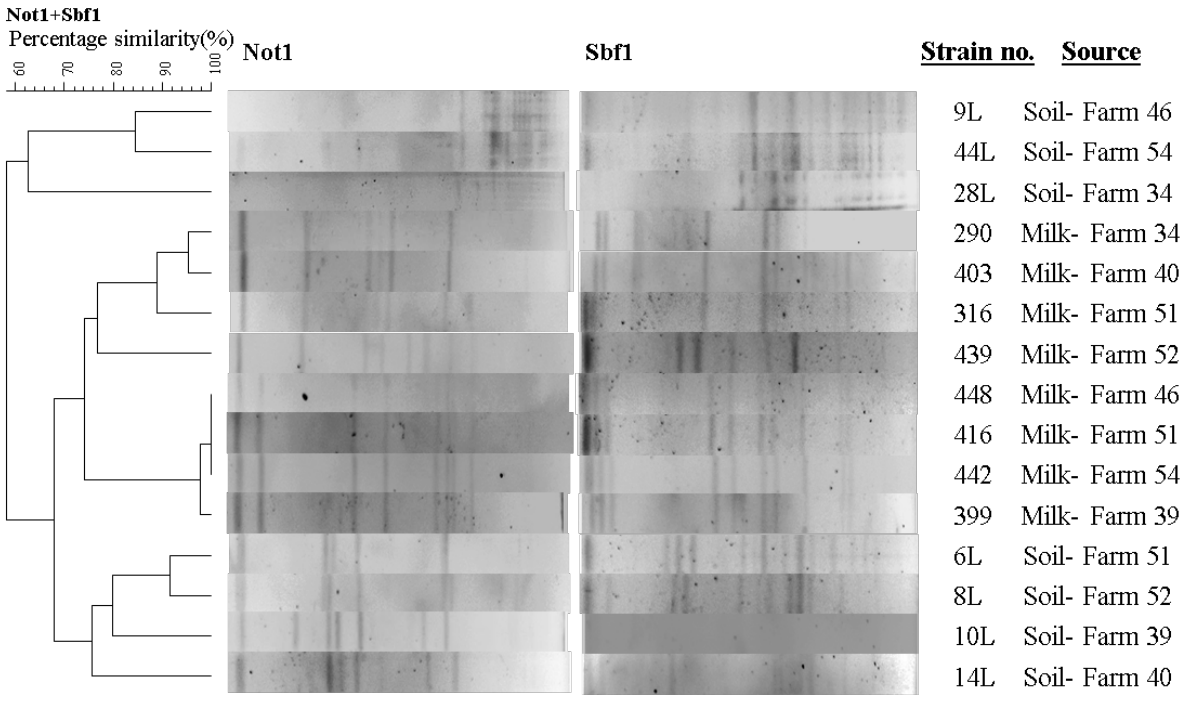

Figure 2. Dendrogram of PFGE pulsotypes of presumptive *Bacillius cereus sensu lato* isolates obtained from soil and milk samples taken from seven farms on the day of the farm visit and on days prior to the visit.

confirmed that the majority of isolates belonged to *B. cereus s.l.* Presumptive *B. cereus s.l.* were identified in all sample sources, including BTM, milk filters, tap water, final rinse water, liners and cow teats on different farms. It is thus apparent that *B. cereus s.l.* organisms are widely distributed in the farm environment, and it is likely that a number of contamination sources of BTM exist. Isolates from the soil did not have the typical colony morphology on BACARA agar and instead had a rhizoidal growth pattern. Nevertheless, these isolates were tentatively identified as presumptive *B. cereus s.l.* by 16S rRNA sequencing. Strain similarity between milk samples from different farms was demonstrated by PFGE analysis. However, no similarity was seen between soil and milk isolates from each farm using this method. Substantially more sampling may be required on each farm to establish shared pulsotypes between milk and the farm environment.

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