Seasonal occurrence and molecular diversity of clostridia species spores along cheesemaking streams of 5 commercial dairy plants

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ABSTRACT

Five commercial dairy plants were monitored over a 17-mo period to determine the seasonal occurrence of *Clostridium* spores in streams from the cheesemaking process. Every 2 mo, samples of raw milk (RM), separated cream (SC), pasteurized and standardized vat milk (PSVM), PSVM + lysozyme (PSVM+L), and manufactured cheese aged for 60 to 90 d were processed for analysis. Molecular diversity of the main species identified was determined using repetitive element palindromic PCR. The mean anaerobic spore counts ($\mu \pm \text{SE}$) were 3.16 ± 0.054 , 3.00 ± 0.054 , 2.89 ± 0.059 , and $2.03 \pm 0.054 \log_{10}$ most probable number/L for RM, PSVM, PSVM+L, and SC, respectively. Although spore counts did not differ between dairy plants, seasonal variation was observed; spore counts of RM, PSVM, and PSVM+L were higher during winter (June to August) and summer (December to February) months, but no seasonal variation was seen in SC counts. The most frequently isolated species was *Clostridium tyrobutyricum*, ranging from 50 to 58.3% of isolates from milk and cream samples. *Clostridium sporogenes* was the second most common species identified (16.7–21.1%); *Clostridium beijerinckii* and *Clostridium butyricum* were also found, although at lower prevalence (7.9–13.2%). Analysis of the *C. tyrobutyricum* and *C. sporogenes* population structure through repetitive element palindromic PCR indicated a high diversity, with unique isolates found in each positive sample. The occurrence of *Clostridia* spores in incoming streams to cheesemaking was most prominent in the winter and summer seasons, with higher prevalence of *C. tyrobutyricum* in the months of June and August.

Key words: *Clostridium*, seasonal variation, identification, cheese late blowing, repetitive element palindromic PCR

INTRODUCTION

Clostridium is a diverse genus of obligate anaerobic, endospore-forming, and gram-positive rod-shaped bacteria that are commonly found in the dairy environment (McAuley et al., 2014). Late blowing is one of the most serious and economically important defects caused by clostridia in hard and semi-hard cheeses. This defect may appear 1 to 2 mo after the cheese is manufactured and is caused by the outgrowth of clostridia species able to convert lactic acid to butyric acid, carbon dioxide, and hydrogen at relatively low pH (Le Bourhis et al., 2005; Vissers et al., 2006). Late blowing can lead to major effects on product quality and commercial value, and produces the deformation of cheese loaf with presence of irregular eyes, slits, or cracks that may lead to downgrading of the cheese, accompanied by off-flavors after several weeks or months of ripening. Although *Clostridium tyrobutyricum* is the most frequently isolated species from late blown cheeses, spores of other clostridia, particularly *C. sporogenes*, *C. beijerinckii*, *C. butyricum*, *C. bifermentans*, *C. perfringens*, and *C. tertium*, have also been isolated from natural and processed cheeses and raw milk (Le Bourhis et al., 2007; Feligini et al., 2014; Reindl et al., 2014), but they are generally regarded as enhancers of the late-blowing defect (Klijn et al., 1995; Le Bourhis et al., 2007).

Julien et al. (2008) identified the presence of clostridia in various components of the milk production chain and *Clostridium tyrobutyricum* was consistently found in raw milk and was widespread in all farm environments examined. The most common contamination sources in the farm are soil, silage and other feeds, and feces, which are transferred to the raw milk via dirt contamination of cow teats during milking. In fact, poor quality of silage can be the main external source of contamination of raw milk by clostridia spores (Vissers et al., 2006, 2007b; Julien et al., 2008). When dairy cows are fed with silage and other feeds, spores are concentrated in the gastrointestinal tract and may cross-contaminate the milk during the milking process, especially when

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improper hygiene practices fail to minimize the spores present on teat surfaces (Visser et al., 2007c). In the last decade, dairy farmers in many parts of the world such as Oceania and South America have been feeding their lactating cows a higher proportion of silage, hay, and concentrate relative to grazing, to increase milk productivity and also to provide greater management flexibility. As a consequence of milk production intensification, many cheesemaking plants are experiencing open texture problems including unwanted slits/cracks in cheeses due to gas production. In addition, in other countries a contamination above 1,000 spores/L of bulk tank milk has a direct economic consequence for farmers because payment depends on this criterion (Visser et al., 2007a,c).

At a farm level, spore contamination of raw milk can be controlled by improving cow hygiene, dairy farm environment, and good milking practice (Doyle et al., 2015). At the dairy factory, the amount of sporeformers in cheese milk may be reduced by bacteriostatic or microfiltration (0.8–1.8 μm), or inhibited by additives such as sodium nitrate, lysozyme, nisin, or reuterin, but their use can be restricted by local regulations (Ávila et al., 2014). Currently, utilization of lysozyme (muramidase, EC 3.2.1.17) is a widespread adopted practice in cheese factories for hard and semi-hard cheese production.

The objectives of this study were to (1) examine the effect of seasonal variation on *Clostridium* spore-former counts in raw milk supplied to 5 commercial dairy plants; (2) to investigate the associated changes in this distribution through processing, i.e., pasteurization, cream separation, and addition of lysozyme; and (3) to identify the main clostridia contaminant species, their prevalence, and their associated population structure including associations of species subgroups with through-chain niches.

MATERIALS AND METHODS

Cheese Plant Selection and Sampling Sites

Five cheesemaking plants (**ST1**, **ST2**, **ST3**, **HT1**, **HT2**) operating in south and southwest Uruguay were selected for microbiological monitoring of milk and cream during the cheese manufacturing process. Dairy factories included in the study had an overall intake of 77% annual raw milk production of the country (INALE, 2014a). Cows were fed under strip-grazing year round by providing a fresh strip of pasture daily. The pooled ration of the dairy cows included 19 to 24% forage reserves (Chilibroste, 2015), and a high proportion (90%) of the annual forage reserves consisted of silage,

mainly sorghum and corn (INALE, 2014b), and two-thirds (65%) of silage was consumed during winter and summer. Swiss-type (**ST**) cheeses were manufactured in plants **ST1**, **ST2**, and **ST3** and Italian hard type (**HT**) cheeses in plants **HT1** and **HT2**, using bovine milk. Duplicate samples were collected every 2 mo at different stages of the manufacture process over a 17-mo study period (April 2013 to August 2014). Samples were collected directly from the processing facilities at the sampling points as follows: raw milk (**RM**), separated cream (**SC**) collected from raw milk separation at 50°C, pasteurized (plate exchanger **HTST**, 72°C for 15 s) and standardized vat milk (**PSVM**), **PSVM** + lysozyme (25 g/1,000 L, **PSVM+L**) sampled before rennet addition. After collection, all samples were delivered to the laboratory in insulated coolers containing frozen coolpacks and were analyzed immediately upon arrival. Samples were transported to the laboratory within 10 h of sampling. Swiss and Italian type cheeses processed using **PSVM+L** from the sampled vats were also analyzed after a period of ripening (2–3 mo) in the dairy plant. Twenty-eight cheeses (Swiss type, $n = 17$; and Italian type, $n = 11$) randomly selected from the previous process were used by cutting a loaf (~3 cm wide) across the middle of the wheel, and eliminating ~3 cm from the rind. Samples were then processed for isolation and enumeration of clostridia species as described below.

Enumeration, Isolation, and Characterization of Anaerobic Sporeforming Bacteria from Milk and Cheese

Clostridium spore concentration was quantified by the most probable number (**MPN**) procedure using a 3×3 scheme. Serial 10-fold dilution of **RM**, **PSVM**, **PSVM+L**, and **SC** samples was prepared in 9 mL (0.1% wt/vol) of sterile peptone water. Samples of **SC** and cheese were previously homogenized in a laboratory blender Stomacher 400 Circulator (Seward Ltd., Worthing, UK) as follows: (1) cream samples (~80 mL) were treated for 2 min at 260 rpm to disrupt fat globules, and (2) 10 g of each representative cheese sample was treated in 90 mL of sterile 2% (wt/vol) trisodium citrate solution preheated at 40°C (2 min at 260 rpm) and the homogenate immediately filtered through sterile Whatman paper no. 4. One-milliliter aliquots of undiluted sample or decimal dilution were inoculated into each of 3 tubes containing 9 mL of Reinforced Clostridium Media (**RCM**, Oxoid, Basingstoke, UK) broth. The inoculated tubes were then sealed with a sterile layer of paraffin:vaseline (1:5), about 15 mm thick, and heated for 10 min at 80°C to inactivate vegetative cells and to

trigger the germination of spores. Tubes were incubated at 37°C for 7 d and observed daily for gas production. For each gas-positive sample, a loopful of one tube was plated on Reinforced Clostridium Agar (Oxoid) containing 50 µg of neutral red (Sigma, St. Louis, MO) and 200 µg of D-cycloserine (Sigma) per mL (Jonsson, 1990) and incubated at 37°C for 72 h in anaerobic jars using the Anoxomat MARK II gas exchange system (MART Microbiology B.B., the Netherlands) with a gas mixture of 85% N₂, 10% CO₂, and 5% H₂. After incubation, up to 3 yellow colonies were randomly picked out from the plate and purified by streaking on RCM (Oxoid) agar. Pure isolates were stored in RCM (Oxoid) with 15% glycerol at -80°C for further identification.

Phenotypic characterization was performed by routine laboratory test and isolates were analyzed by observing colony morphology, catalase and oxidase test, Gram staining, ability to grow aerobically on RCM (Oxoid, UK), and spore presence and position using phase contrast microscope (Olympus Cx41) with digital camera. Only gram-positive obligate anaerobic endospore forming isolates were further studied.

Isolation of Genomic DNA

Presumptive *Clostridium* isolates were grown overnight in test tubes with RCM (Oxoid) broth, previously filtered to remove agar, under anaerobic conditions at 37°C. Bacterial cells were harvested at 7,200 × *g* for 5 min at 4°C in a Spectrofuge 7M tabletop centrifuge (Labnet International Inc., Edison, NJ), and cell pellets were suspended in 200 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was purified using a Genomic DNA purification kit (ZR Fungal/Bacterial DNA MiniPrep, Zymo Research, Irvine, CA) following the manufacturer's instructions. Purified DNA was suspended in 40 µL of TE buffer and used as template in amplification reactions. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Isolate Identification by Multiplex-PCR and 16s rDNA Sequence Analysis

Occurrence of the main species involved in late blowing were analyzed by multiplex PCR using 4 species-specific primer pairs for *C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii*, and *C. sporogenes* in accordance with Cremonesi et al. (2012). Specific multiplex PCR primers (Macrogen, Seoul, Korea) were resuspended to a final concentration of 100 pmol/µL in sterile double-distilled water. The PCR was performed in a 50-µL reaction mixture containing, 1.25 U of Taq polymerase

(Fermentas, Waltham, MA), 1× Thermo buffer (Fermentas), 3 mM MgCl₂, 200 µM of each dNTP (Fermentas), the primer pair amount according to Cremonesi et al. (2012), and 40 ng of template DNA. Reactions were carried out in a Corbett CG1-96 thermal cycler with a palmtop computer interface (Corbett Research Ltd., Cambridge, UK) with the following conditions: initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. Final extension was carried out at 72°C for 5 min. The amplified PCR products were distinguished by gel electrophoresis in a 3% agarose gel, stained with GoodView Nucleic Acid Stain 5% (vol/vol; SBS Genetech Co. Ltd., Beijing, China) and visualized by UV transilluminator. A 100-bp DNA ladder (Generuler 100 bp DNA Ladder Plus, Fermentas) was included in each gel electrophoresis. Other anaerobic spore-formers yielding negative results by the multiplex PCR were further speciated by a 16s rRNA sequencing region using universal eubacterial primers fd1 and rd1 (Weisburg et al., 1991). Briefly, 25-µL PCR reaction mixtures contained 1× Thermo buffer (Fermentas), 2.5 mM MgCl₂, 200 µM of each dNTP (Fermentas), 1 U of Taq polymerase (Fermentas), 0.2 mM of each PCR primer (fd1 and rd1), and 20 ng of template DNA. Primers fd1 and rd1 were used to amplify a 1,540-bp genome fragment with 16S rRNA gene sequences. The PCR were performed using an initial denaturation step at 94°C for 7 min, then 35 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min, and experiments included negative controls with no added DNA template. Amplified fragments were purified and sequenced by Macrogen Sequencing Service, Korea, using an ABI Prism3730XL capillary sequencer (Applied Biosystems, Foster City, CA). The DNA sequences were compared with those of the NCBI BLAST database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify type strains with highest similarity.

Genomic Fingerprinting Analysis Using Repetitive Element Palindromic PCR (GTG)₅

The 2 most common species identified, *C. tyrobutyricum* and *C. sporogenes*, were subjected to repetitive element palindromic PCR (**rep-PCR**) genomic fingerprinting analysis using PCR primer (GTG)₅ to study intra-species diversity (Versalovic et al., 1994). Analysis was carried out in 25-µL reaction mixtures containing 1× Thermo buffer (Fermentas), 1.5 mM MgCl₂, 200 µM of each dNTP (Fermentas), 1 U of Taq polymerase (Fermentas), 2 µM (GTG)₅ primer (Gevers et al., 2001), and 20 ng of template DNA. Amplifica-

tions were performed in a DNA thermal cycler Corbett CG1-96 with a palmtop computer interface (Corbett Research Ltd.). The PCR cycling parameters included a denaturation step at 95°C for 7 min; 35 cycles each consisting of 94°C for 1 min, 46°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 10 min. Control reaction mixtures lacking template DNA were included with each analysis. The PCR products were electrophoresed on 1.5% agarose gels using 0.5× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH = 8.0) as running buffer at 10 V/cm for 1 h, stained with GoodView Nucleic Acid Stain 5% (vol/vol; SBS Gene-tech Co. Ltd.), and visualized and photographed on a UV transilluminator. As a size standard, the Generuler 100-bp DNA Ladder Plus (Fermentas) was used. Band profiles were visualized and photographed under UV light using an ultra-compact gel documentation system (Cleaver Scientific Ltd., Warwickshire, UK).

Statistical Analysis

The MPN data of anaerobic spore counts obtained during the study were transformed to $\log_{10}$ for statistical analysis. Data analysis for cheese milk was performed using a statistical model as follows:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{1ij} + \delta_k + \gamma_{jk} + \varepsilon_{2ijk},$$

where y_{ijk} is the response variable, μ is the overall mean, α_i is the effect of the i th dairy plant, β_j is the effect of the j th sampling point, ε_{1ij} is the experimental error associated with dairy plant and sampling point, δ_k is the effect of the k th month of sampling, γ_{jk} is the effect of interaction the j th sampling point and the k th date of sampling, and ε_{2ijk} is the experimental error associated with date of sampling. The model was adjusted with GLIMMIX of SAS Ver. 9.4 (2014, SAS Institute Inc., Cary, NC). Overall mean of anaerobic spore counts by season were analyzed using the ESTIMATE SAS statement. Mean differences of the pooled spore counts data by season were analyzed by using the t-test.

Cheese spore counts ($\log_{10}$ MPN/g) were analyzed using a mixed model estimated by restricted maximum likelihood as follows:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \alpha_i \times \beta_j + \delta_k(\alpha_i) + \varepsilon_{ijkl},$$

where y_{ijk} is the response variable, μ is the overall mean, α_i is the effect of the i th cheese type, β_j is the effect of the j th sampling month, $\alpha_i \times \beta_j$ is the cheese type $\times$ sampling month interaction term, $\delta_k(\alpha_i)$ is the effect of plant within cheese type, and ε_{ijkl} is the residual error.

The model was analyzed by PROC GLIMMIX of SAS Ver. 9.4.

The genomic data (GTG)₅-PCR fingerprintings were analyzed by Gel Compar II 6.5 software (Applied Maths, Kortrijk, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation coefficients with the unweighted pair group method with arithmetic mean clustering algorithm (UPGMA) analysis method.

RESULTS AND DISCUSSION

Seasonal Variation of Anaerobic Spore Counts in Raw and Vat Milk

The mean anaerobic spore counts ($\mu \pm \text{SE}$) recorded during the study were 3.16 ± 0.054 , 3.00 ± 0.054 , 2.89 ± 0.059 , and $2.03 \pm 0.054 \log_{10}$ MPN/L for RM, PSVM, PSVM+L, and SC, respectively. The results for RM, PSVM, and PSVM+L were within the range of data previously reported (Visser et al., 2007a; Zucali et al., 2011; Arias et al., 2013), although other studies revealed lower levels of spores (Colombari et al., 2001). The incidence of anaerobic spore counts did not differ significantly ($P = 0.3945$) between dairy plants. Statistical analysis of the data showed a significant ($P = 0.0226$) effect in the interaction between month of sampling and sampling point, indicating that differences between RM, PSVM, PSVM+L, and SC depends on the month of sampling. Figure 1 gives an overview of the seasonal pattern of anaerobic spore counts in RM, PSVM, PSVM+L, and SC during the period of study, with significant ($P < 0.05$) lower mean values in RM, PSVM, and PSVM+L during autumn and spring months; however, no differences ($P > 0.05$) were found associated with SC (Table 1). As expected, the spore counts of milk samples were higher during the winter and summer season, when dairy cows from farms supplying dairy factories in this study were supplemented with higher amount of silage in the total diet. In recent years, year-round grazing systems in Uruguay have undergone an accelerated process of intensification (Chilibroste, 2015), with major changes in silage feeding, and dairy farmers were forced to supplement during winter and summer. To this end, control of microbial load in feeds such as silage may be central to lowering the incidence in the milk supply to dairy plants in this study (Visser et al., 2007c).

The negligible effect of lysozyme on spore counts in the cheese vat was likely due to insufficient time for spore germination after HTST treatment coupled with the associated dosage in the cheese plant. Spores

Table 1. Most probable number clostridia spore enumeration from raw milk (RM), pasteurized and standardized vat milk (PSVM), PSVM added with lysozyme (PSVM + L), and separated cream (SC) samples, across the course of the study¹

Sampling date	RM	PSVM	PSVM + L	SC
April 13	2.85 ^{bcd,A}	2.79 ^{bcd,A}	2.65 ^{bc,A}	2.01 ^{b,B}
June 13	3.30 ^{ab,A}	3.01 ^{abcd,A}	3.00 ^{ab,A}	1.45 ^{d,B}
August 13	3.27 ^{ab,A}	3.00 ^{abcd,AB}	2.79 ^{abc,B}	2.02 ^{b,C}
October 13	2.68 ^{d,A}	2.59 ^{d,A}	2.43 ^{c,A}	1.96 ^{bc,B}
December 13	3.36 ^{a,A}	3.18 ^{ab,A}	3.12 ^{ab,A}	1.55 ^{cd,B}
February 14	3.26 ^{abc,A}	3.05 ^{abc,A}	2.89 ^{abc,A}	2.54 ^{a,B}
April 14	2.81 ^{cd,A}	2.73 ^{cd,A}	2.70 ^{bc,A}	2.07 ^{b,B}
June 14	3.39 ^{a,A}	3.34 ^{a,A}	3.13 ^{ab,A}	2.31 ^{ab,B}
August 14	3.52 ^{a,A}	3.31 ^{a,A}	3.26 ^{a,A}	2.38 ^{ab,B}

^{a-d}Different letters in the same column indicates significant differences ($P < 0.05$).
^{A-C}Different letters in the same row indicates significant differences ($P < 0.05$).
¹Spore counts are presented as log₁₀ spores per liter.

are completely resistant to lysozyme (Wasserfall and Teuber, 1979).

Relative Abundance of Main Clostridia Species in Raw and Vat Milk

A total of 260 isolates were collected from RM (n = 72), PSVM (n = 89), PSVM+L (n = 61), and SC (n = 38) samples. Occurrence of the main species in RM, PSVM, PSVM+L, and SC are presented in Table 2. *Clostridium tyrobutyricum* was the most frequently isolated species throughout the study ranging from 50 to 58.3% of isolates from milk and cream samples, with peaks in isolation frequency in winter and summer months. *Clostridium sporogenes* was the second most frequently isolated species (16.7–21.1% of total isolates from samples) and showed to be more evenly distributed across all sampling months. Nevertheless, this finding is not in agreement with Feligini et al. (2014) who found that *C. sporogenes* were higher in winter and *C. tyrobutyricum* were higher during winter and spring.

Figure 1. Seasonal distribution of anaerobic spores (log₁₀ MPN/L) in raw milk (RM), pasteurized and standardized vat milk (PSVM), PSVM added with lysozyme (PSVM+L), and separated cream (SC) from 5 cheesemaking plants along the period of study. Error bars are SE of the samples grouped by season.

Clostridium beijerinckii and *C. butyricum* were also found, though at lower prevalence (from 7.9 to 13.2%). *Clostridium butyricum* showed a decreased occurrence in SC, likely because it was more easily removed by centrifugal force than *C. tyrobutyricum*, *C. sporogenes*, and *C. beijerinckii*, due to a smaller spore size (Su and Ingham, 2000). Within *Clostridium* sp. several minor species were identified, and the most frequently isolated in this group were *C. bifermentans* and *C. acetobutylicum*, representing 4.2% and 2.7% of the total isolates analyzed, respectively; the former previously isolated from milk and silage samples in other studies (Jonsson, 1990; Reindl et al., 2014). Other species identified by 16s rRNA sequentiation were *C. xylanolyticum* and *C. aminovalericum*.

Table 2. Relative occurrence of the main anaerobic spore-formers in RM, PSVM, PSVM + L, and SC during the period of study

Species	RM ¹		PSVM		PSVM + L		SC	
	Isolates (no.)	%	Isolates (no.)	%	Isolates (no.)	%	Isolates (no.)	%
<i>Clostridium tyrobutyricum</i>	42	58.3	46	51.7	31	50.8	19	50.0
<i>Clostridium sporogenes</i>	12	16.7	17	19.1	12	19.7	8	21.1
<i>Clostridium butyricum</i>	5	6.9	9	10.1	6	9.8	1	2.6
<i>Clostridium beijerinckii</i>	6	8.3	7	7.9	5	8.2	5	13.2
<i>Clostridium</i> spp.	7	9.7	10	11.2	7	11.5	5	13.2

¹Sampling point in the dairy stream: raw milk (RM), pasteurized and standardized vat milk (PSVM), pasteurized and standardized vat milk added with lysozyme (PSVM + L), separated cream (SC) during standardization process.

These results are in good agreement with the findings of Bergère and Accolas (1986), Reindl et al. (2014), and Matteuzzi et al. (1977) who found that both species (*C. tyrobutyricum* and *C. sporogenes*) showed the highest prevalence in milk and cheese samples, accounting for 75 and 84.7%, and 92.5% of analyzed samples, respectively. Spores of *C. sporogenes*, *C. beijerinckii*, and *C. butyricum* have also been isolated from milk and natural or processed cheeses (Le Bourhis et al., 2007). The importance of silage in this contamination issue is highlighted by Jonsson (1990), who showed a high association of *C. tyrobutyricum* in the raw milk of silage-fed dairy cows.

Incidence of Clostridium Spores in Manufactured Cheeses

Twenty-eight manufactured vats of Swiss type ($n = 17$) and Italian type ($n = 11$) cheeses were monitored and analyzed between 60 to 90 d of ripening. Of these, only 1 Swiss type cheese out of 17 presented with late blowing; this cheese was produced in winter, when spore counts were among the highest recorded. None of the Italian type manufactured cheeses displayed this defect. These results support the efficacy of lysozyme as a preservative to prevent spore outgrowth by clostridia, which may lead to late blowing. Similar results by Bassi et al. (2015) demonstrated that lysozyme showed greater activity against *C. tyrobutyricum*, in favor of a relatively higher prevalence of *C. butyricum*. Swiss type cheeses are particularly susceptible to late blowing because growth of causative bacteria may be favorably influenced by the duration and temperature (20–25°C) used in the warm room step of ripening, a relatively higher pH and moisture content, and lower salt content (Dasgupta and Hull, 1989; Le Bourhis et al., 2007). Spore counts of Swiss type cheeses (1.49 ± 0.13 MPN/g) were higher ($P = 0.0345$) than Italian type cheese (0.74 ± 0.13 MPN/g), likely due to these lower associated stress factors inhibitory to *Clostridium* growth. Seasonal behavior showed differences ($P = 0.0215$) in clostridia counts in cheese samples, with higher levels recorded in winter and summer months. Twenty-eight isolates were obtained among all Swiss type cheese samples tested, and were identified as follows: 78.6% were *C. tyrobutyricum*, 14.2% *C. sporogenes*, and 7.2% *C. butyricum*. *Clostridium tyrobutyricum* was the prevalent anaerobic spore-former species in ST cheese, even when the spore concentration was low. No *C. tyrobutyricum* or *C. sporogenes*, however, were isolated from HT cheeses. The only species isolated from the cheese showing late blowing was *C. tyrobutyricum*.

Differentiation of *C. tyrobutyricum* and *C. sporogenes* Isolates by (GTG)₅-PCR Fingerprint Analysis

The rep-PCR analysis dendrogram obtained for 44 isolates of *C. tyrobutyricum* is shown in Figure 2. Clade T1 contains a diverse set of 11 isolates, all from plants producing Italian hard cheeses, which cluster distinct from other isolates. This suggests these plants may have a genetically distinct subpopulation of *C. tyrobutyricum* isolates. Interestingly, isolates from this group were only identified in RM or PSVM, suggesting isolates clustering in this subpopulation may show higher sensitivity to lysozyme. This reflects recent findings by Ávila et al. (2014), who identified *C. tyrobutyricum* as the most sensitive species to lysozyme among clostridia species tested. Lysozyme hydrolyzes the β -1,4 linkages of the peptidoglycan layer of the cell wall leading to cell lysis; however, its efficacy varies against different clostridia species, and against spores or vegetative cells (with the latter usually showing greater sensitivity; Ávila et al., 2014). Four of the other 5 clades identified (with the exception of clade T3), contained isolates from HT and ST cheese plants, suggesting a widespread distribution of these subtypes. Clade T3, however, contained only isolates from ST plants with 73% of these isolated from cheese, and a single isolate from PVSM + L. This grouping also showed the highest degree of similarity (>95%).

The dendrogram of the 46 *Clostridium sporogenes* isolates are presented in Figure 3. Neither HT nor ST cheese plants appeared to harbor distinct subpopulations, with the exception of clade S3, which contained 4 milk isolates from a single HT plant (HT2).

An overall high biodiversity was identified among both *C. tyrobutyricum* and *C. sporogenes* populations. Similar high diversity among clostridia species populations has also been reported in other studies (Garde et al., 2012; Nishihara et al., 2014), suggesting dairy chains may often feature a diverse clostridia species population, and attributing late blowing to individual strains or tracking contamination routes may be difficult, requiring extensive isolate sampling.

CONCLUSIONS

Results of this study suggest seasonal patterns in the prevalence of anaerobic sporeformers in the Uruguayan dairy chain, with higher concentrations in raw and vat milk during winter and summer months. Pasteurization, standardization, or addition of lysozyme did not significantly reduce the clostridia population during processing. *Clostridium tyrobutyricum* and *C.*

sporogenes were the most abundant species in raw and vat milk, and in cheeses with or without late blowing symptoms. Although a high clostridia prevalence was identified among milk samples, the overall incidence of late blowing in Swiss and Italian type cheeses was low, which may be due to the use of lysozyme to control clostridia outgrowth. Both *C. tyrobutyricum* and *C. sporogenes* populations showed high molecular diversity, suggesting the on-farm populations to be equally diverse. Identification of points of contamination at the

farm level, and further understanding of the physiological and genetic characteristics of subpopulations may provide key inputs to control strategies against these organisms for the dairy industry.

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Figure 2. Dendrogram based on cluster analysis of (GTG)₅-PCR fingerprinting profiles of 44 isolates of *Clostridium tyrobutyricum*. Isolate = identification number of the isolate; plant = identification of the 5 dairy plants manufacturing Swiss type (ST1, ST2, ST3) and Italian Hard type (HT1, HT2) cheese (CH); sample = raw milk (RM), pasteurized and standardized vat milk (PSVM), PSVM added with lysozyme (PSVM + L), and separated cream (SC); month = month of sampling of the isolate. T1, T2, T3, T4, T5, and T6 = denomination of *C. tyrobutyricum* clades.

Figure 3. Dendrogram based on cluster analysis of (GTG)₅-PCR fingerprinting profiles of 46 isolates of *Clostridium sporogenes*. Isolate = identification number of the isolate; plant = identification of the 5 dairy plants manufacturing Swiss type (ST1, ST2, ST3) and Italian Hard type (HT1, HT2) cheese (CH); sample = raw milk (RM), pasteurized and standardized vat milk (PSVM), PSVM added with lysozyme (PSVM + L), and separated cream (SC); month = month of sampling of the isolate. S1, S2, S3, S4, and S5 = denomination of *C. sporogenes* clades.

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