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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 ($\mu \pm$ SE) were 3.16 ± 0.054, 3.00 ± 0.054, 2.89 ± 0.059, and 2.03 ± 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 contamina-

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tion 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 improper hygiene practices fail to minimize the spores present on teat surfaces (Vissers 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 (Vissers 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 bactofugation 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* sporeformer 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 the south and the south-west of 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