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Enumeration of clostridia in goat milk using an optimized membrane filtration technique

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ABSTRACT

A membrane filtration technique developed for counting butyric acid bacteria in cow milk was further developed for analysis of goat milk. Reduction of the sample volume, prolongation of incubation time after addition of proteolytic enzyme and detergent, and a novel step of ultrasonic treatment during incubation allowed filtration of goat milk even in the case of somatic cell counts (SCC) exceeding 10^6/mL. However, filterability was impaired in milk from goats in late lactation. In total, spore counts were assessed in 329 farm bulk goat milk samples. Membrane filtration technique counts were lower than numbers revealed by the classic most probable number technique. Thus, method-specific thresholds for milk to evaluate the risk of late blowing have to be set. As expected, the spore counts of milk samples from suppliers not feeding silage were significantly lower than the spore counts of milk samples from suppliers using silage feeds. Not only were counts different, the clostridial spore population also varied significantly. By using 16S rRNA gene PCR and gene sequencing, 342 strains from 15 clostridial species were identified. The most common *Clostridium* species were *Clostridium tyrobutyricum* (40.4%), *Clostridium sporogenes* (38.3%), *Clostridium bifermentans* (7.6%), and *Clostridium perfringens* (5.3%). The 2 most frequently occurring species *C. tyrobutyricum* and *C. sporogenes* accounted for 84.7% of the isolates derived from samples of suppliers feeding silage (n = 288). In contrast, in samples from suppliers without silage feeding (n = 55), these species were detected in only 45.5% of the isolates.

Key words: clostridia, butyric acid bacteria, goat milk, membrane filtration, PCR

INTRODUCTION

Clostridium is a diverse genus of obligate anaerobic, endospore-forming, and gram-positive microorganisms. Pathogenic species such as *Clostridium botulinum*, *Clostridium difficile*, *Clostridium tetani*, and *Clostridium perfringens* produce up to 18% of all known bacterial toxins, thus making *Clostridium* the most toxic prokaryotic genus and a significant concern to human and animal health (Popoff and Stiles, 2005). However, the food-related pathogens *C. botulinum* and *C. perfringens* are of negligible importance to the dairy industry. Two outbreaks of botulism related to Brie cheese (Sébald et al., 1974; Johnson et al., 1990) and Mascarpone cheese (Aureli et al., 1996, 2000) were associated with elevated pH values in the product and temperature abuse. Botulism from hazelnut yogurt was traced to toxin contained in nut purée but not in the milk component (O'Mahony et al., 1990).

Nevertheless, spores of clostridia are a challenge for the dairy industry as major spoilage organisms of hard and semi-hard cheeses. A serious defect predominantly in Emmental cheese is the formation of white putrid spots in the interior of the cheese caused by intense proteolysis by *Clostridium sporogenes* (Hüfner, 2001; Bachmann et al., 2011). *Clostridium oceanicum* and *C. sporogenes* may also provoke proteolysis on the surface of film-matured cheeses, causing white taints on the cheese surface accompanied by an intense putrid odor (Hüfner, 2001). The most important defect, however, is butyric acid fermentation (**BAF**) by *Clostridium tyrobutyricum*, which converts lactic acid to butyric acid, acetic acid, CO_2, and hydrogen. This defect causes formation of cracks, abnormally shaped or excessively big eyes, or even blowing of the cheese, accompanied by off-flavors after several weeks or months of ripening. Thus, the defect is referred to as "late blowing." Other clostridia (e.g., *Clostridium beijerinckii*, *C. sporogenes*, *Clostridium butyricum*, and *Clostridium bifermentans*) were also detected in defective cheeses, but they are considered as enhancers of the late-blowing defect (Klijn et al., 1995; Le Bourhis et al., 2007; Daly et al., 2010). However, all these species as well as *Clostridium*

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cochlearium may spoil processed cheese products that have been gently heated (Stadhouders et al., 1985; Lycken and Borch, 2006) because the surviving spores are able to germinate and grow due to the presence of lactose and the relatively high pH values of 5.5 to 6.0.

The main source of clostridial spores in raw milk is silages of insufficient microbial quality. The spores are concentrated in the feces of the lactating animals and enter the milk during milking, especially when improper hygiene practices fail to minimize the spores present on the teat surfaces (Stadhouders et al., 1985; Vissers et al., 2007; Sheehan, 2011).

Preventive measures to control BAF are (1) avoiding milk contamination by prohibiting use of silage as a feed for lactating ruminants, which is practiced in some European regions for the manufacture of cooked hard cheeses; (2) ensuring good hygiene practices in animal husbandry and during the milking process; (3) removing spores from cheese milk through bactofugation or microfiltration; and (4) using clostridial growth inhibitors such as nitrate or lysozyme (Stadhouders et al., 1985; Bachmann, 1995; Sheehan, 2011).

In recent years, cheeses produced from goat milk are of increasing commercial relevance, and the late-blowing defect was also observed in these cheeses even when processing milk from farms without silage feeding. However, data of the clostridial load in goat milk have not been available until now.

Control of BAF in cheese needs reliable methods to enumerate the relevant clostridial spores in farm bulk milk or in cheese milk before processing. Thus, methods must be available for counting lactate fermenting butyric acid bacteria (**BAB**), essentially *C. tyrobutyricum*. As few as 10 to several hundred spores of BAB per liter of milk may induce BAF (Freyer and Halligan, 1976; Bergère and Sivelä, 1990; Sheehan, 2011). Therefore, most probable number (**MPN**) techniques are used including the possibility of detecting the spores in up to 10-mL sample volumes. For milk of cows fed a silage-free diet, a threshold of 200 BAB spores per liter is recommended (Freyer and Halligan, 1976; Teuber, 1985). Unfortunately, neither an international standard method exists nor any routine method is able to count specifically the relevant species *C. tyrobutyricum* (Zangerl, 1989; Bergère and Sivelä, 1990).

The methods differ in the choice of enrichment broths, pasteurization conditions of milk samples to eliminate the vegetative cells, and incubation times. The most important means to achieve sufficient selectivity for BAB are the presence of lactate as available carbon source and adjustment of pH of the broth to levels prevalent in hard and semi-hard cheeses (pH 5.4–5.5). In the Netherlands and in Austria, a milk medium containing glucose and lactate adjusted to pH 5.45 is used [NIZO method (NIZO food research BV, Ede, the Netherlands) according to van den Berg et al. (1988)]. Recently, this method was laid down as the national standard in the Netherlands in a slightly modified way (NEN, 2009). In Germany, reinforced clostridial medium (**RCM**) adjusted with lactic acid to pH 5.4 is used (VDLUFA, 1996). France and Switzerland prefer a modified Bryant and Burkey broth containing lactate as the sole fermentable carbon source (CNERNA, 1986; Jakob, 2011). Depending on the method, the pasteurization conditions vary between 75 and 80°C for 5 to 10 min, and incubation times vary between 3 and 7 d at 37°C. Anaerobiosis is achieved by sealing the tubes with paraffin or water agar. The formation of gas, which lifts the paraffin or agar plug, serves as the diagnostic system.

A membrane filtration technique (**MFT**) was developed by Bourgeois et al. (1984) as an alternative to the time- and material-consuming and cumbersome MPN methods. The MFT was modified by Agroscope (Bern, Switzerland). According to Agroscope instructions (unpublished), a 40-mL sample of milk is pasteurized at 76°C for 15 min, incubated with 5 mL of trypsin solution (2% in Tris-HCl) and 2.5 mL of Triton X-100 (16% solution) in a water bath for 15 min at 55°C and filtrated under 250 kPa of pressure through a membrane filter with a pore size of 0.8 μm. The filter is transferred onto a modified reinforced clostridial agar (**RCAm**) containing D-cycloserine to inhibit the growth of facultative anaerobic bacilli (Abgrall and Bourgeois, 1985; Jonsson, 1990) and acid fuchsin dye, supporting the differentiation of colonies (Bergère and Sivelä, 1990; Jakob, 2011). Bächli (1997) mentions a modification by M. Casey and J. Gruskovnjak (unpublished internal report; Agroscope, Bern, Switzerland, 1995) through introducing calcium chloride-2-hydrate, which should enhance germination of *C. tyrobutyricum* spores. Colonies are counted after anaerobic incubation at 37°C for 72 h. Typical *C. tyrobutyricum* colonies are pink to dark red, convex, round, and not fimbriated, with a diameter of 1 to 3 mm. However, when applying this method to goat milk, problems occur with filterability, probably because of the physiologically high SCC (J. Hummerjohann, Agroscope, Bern, Switzerland, personal communication).

Therefore, the aim of the current study was to optimize the MFT for analyzing goat milk. For method validation, the MFT was compared with the NIZO method (van den Berg et al., 1988) both in cow and goat milk samples. The modified procedure was then used on bulk raw milk collected from farmers to assess the BAB spore counts when using silage and nonsilage feeds. Finally, to study the composition of the clostridia population, isolates were typed by partial 16S rRNA