



Effect of jenny milk addition on the inhibition of late blowing in semihard cheese

C. Cosentino,¹ R. Paolino, V. Valentini, M. Musto, A. Ricciardi, F. Adduci, C. D'Adamo, G. Pecora, and P. Freschi

School of Agricultural, Forestry, Food, and Environmental Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10, Potenza 85100, Italy

ABSTRACT

The occurrence of late blowing defects in cheese produces negative effects on the quality and commercial value of the product. In this work, we verified whether the addition of raw jenny milk to bulk cow milk reduced the late blowing defects in semihard cheeses. During cheesemaking, different aliquots of jenny milk were poured into 2 groups of 4 vats, each containing a fixed amount of cow milk. A group of cheeses was created by deliberately contaminating the 4 vats with approximately $3 \log_{10}$ cfu/mL milk of *Clostridium tyrobutyricum* CLST01. The other 4 vats, which were not contaminated, were used for a second group of cheeses. After 120 d of ripening, some physical, chemical, and microbiological parameters were evaluated on the obtained semihard cheeses. Differences in sensory properties among cheeses belonging to the uncontaminated group were evaluated by 80 regular consumers of cheese. Our results showed that the increasing addition of jenny milk to cow milk led to a reduction of pH and total bacterial count in both cheese groups, as well as *C. tyrobutyricum* spores that either grew naturally or artificially inoculated. We observed a progressive reduction of the occurrence of late blowing defects in cheese as consequence of the increasing addition of jenny milk during cheese making. Moreover, the addition of jenny milk did not affect the acceptability of the product, as consumers found no difference among cheeses concerning sensorial aspects. In conclusion, the important antimicrobial activity of lysozyme contained in jenny milk has been confirmed in the current research. It is recommended for use as a possible and viable alternative to egg lysozyme for controlling late blowing defects in cheese.

Key words: cheese late blowing, jenny milk, lysozyme, *Clostridium tyrobutyricum*

INTRODUCTION

Cheeses are susceptible to spoilage of the final product, resulting in an important economic effect on dairy production. The alterations and the defects of curd depend on both technological and microbiological factors. Among technological factors, milk quality, heat treatment, hygiene practices, manufacture technology, compositional parameters, and ripening temperature or moisture are all capable of influencing cheese making and ripening (Matijašić et al., 2007). Microbiological factors are the most difficult to control; undesirable microorganisms, such as coliforms, yeasts, heterofermentative lactic acid bacteria, and spore-forming bacteria may cause early or late blowing defects in cheeses (Little et al., 2008; Gómez-Torres et al., 2014).

Late blowing defects (LBD) on ripened semihard and hard cheese have been attributed to the outgrowth in cheese of strains of *Clostridium* spp., mainly *Clostridium tyrobutyricum*, which is capable of fermenting lactic acid with production of butyric acid, acetic acid, carbon dioxide, and hydrogen (Garde et al., 2012). The pressure of these gases causes cracks and splits that are generally accompanied by unpleasant aroma and rancid flavor. Damaged cheeses also contain several heterogeneously distributed cavities corresponding to the produced gas and to the digested mass (Hughey and Johnson, 1987; Senyk et al., 1989; Kalač, 2011). Late blowing defect is a problem well known to cheese manufacturers, but it is difficult to eradicate because *Clostridium* spores are ubiquitous and much more resistant to heat, chemicals, irradiation, and desiccation than vegetative cells. Moreover, a few spores per liter of milk are enough to induce the defect if cheese conditions are suitable for the germination and growth (Garde et al., 2012).

To demonstrate the causative relationship between *C. tyrobutyricum* and late blowing in cheese, many cheesemaking experiments were made to provoke this defect using several strains spores of the most important dairy-related clostridia (Klijn et al., 1995). *Clostridium tyrobutyricum* spores are highly resistant to environmental conditions and contaminate milk before

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¹Corresponding author: carlo.cosentino@unibas.it

cheese production; in fact, late blowing occurs mostly in cheeses made with unpasteurized milk, even if pasteurized milk cheeses may also be affected (Julien et al., 2008).

The distribution of this problem within the cheese factory is heterogeneous. Contamination may occur in spring and autumn especially, when temperatures are mild and humidity is high, and may affect only some batches or certain pieces within a batch (Garde et al., 2011). It is known that the growth of *Clostridium* is favored by the presence of other microorganisms in cheese, such as coliforms, lactococci, leuconostocs, lactobacilli, and propionibacteria (Anastasiou et al., 2009). Most of the studies on *Clostridium* spp. contamination have been done with cow milk (Visser et al., 2006, 2007a). The main sources of contamination are thought to be silage, water, or unhygienic animal bedding (Julien et al., 2008; Anastasiou et al., 2009). When silage fermentation conditions are not prone to rapid pH decrease and maintenance of uniformly anaerobic conditions, germination of clostridial spores and subsequent vegetative cell multiplication can occur (Visser et al., 2006, 2007b). Other farm environments may also contain clostridia, such as forage surfaces, as endophytic organisms of gramineous plants, in grass and maize silage, in the rumen, manure, milk, and cheese (López-Enríquez et al., 2007; Julien et al., 2008; Bassi et al., 2013).

The most common approaches to prevent this defect include bacteriostatic or microfiltration of milk (Arias et al., 2013), addition of nitrate or lysozyme (Ávila et al., 2014; Medeiros et al., 2014), and addition during cheese manufacture of strains of lactic acid bacteria producing biologically active peptides against gram-positive bacteria (Martínez-Cuesta et al., 2010). Nevertheless, technical limitations exist for these methods: bacteriostatic does not ensure the absence of *Clostridium* spp. and microfiltration requires preskimming of milk (Garde et al., 2011); nitrates may produce potentially carcinogenic nitrosamines (EFSA, 2010); lysozyme from hen eggs may cause allergic problems in egg-hypersensitive people (Frémont et al., 1997; de Roos et al., 1998; Marseglia et al., 2013); and bacteriocins may inhibit the starter development and modify the ripening process of cheese (Leroy and De Vuyst, 2004). Lysozyme, in particular, is a commercial additive extracted from hen egg white (3.5% of the egg white proteins) and is able to lyse the cell walls of the vegetative form of *C. tyrobutyricum* through the enzymatic cleavage in pressed and cooked curds [e.g., Swiss cheese, Grana Padano Protected Designation of Origin (PDO), Edam, Gouda, Cheddar, and many others; Colicin et al., 2004; Dragoni et al., 2011; Cosentino et al., 2013; Marseglia et al., 2013]. This approach is suitable

for certain types of cheese when the concentration of clostridial spores is not too high.

Lysozyme has been approved as a preservative (E1105) in the entire European Community, according to European Parliament Directive No. 95/2/EC (“*quantum satis*” in ripened cheese; Pellegrino and Tirelli, 2000; Scharfen et al., 2007; Schneider et al., 2011). In the recently changed EC legislation, the use of lysozyme as an additive (not exceeding 25 mg/L) has to be declared on the label (EC legislation in Europe 2003/89/EC, Directive 2000/13/EC). It is necessary, therefore, to reliably detect and quantify this preservative in cheese (Iaconelli et al., 2008; Kerkaert et al., 2010; Dragoni et al., 2011). In fact, the residual concentrations of lysozyme in cheese may cause severe allergic reactions in consumers allergic to eggs due to its content in ovomucoid, ovalbumin, and conalbumin (Frémont et al., 1997; Pérez-Calderón et al., 2007; Kerkaert et al., 2010). Those limitations suggest the necessity of applying other control methods to avoid *Clostridium* spp. spores contamination of milk.

Currently, the use of natural additives as food preservers has become popular because of greater consumer awareness and increasing allergies to synthetic chemical additives (Librán et al., 2013). Much interest exists in developing new methods for making food safe and more natural. Recently, Galassi et al. (2012) and Cosentino et al. (2013) described the addition of jenny milk as a substitute for egg lysozyme to prevent late blowing in Grana Padano PDO cheese and ewe cheese, respectively. Jenny milk is characterized by a high lysozyme content (Chiavari et al., 2005; Polidori and Vincenzetti, 2007; Cosentino et al., 2012a,b), and an array of defense protein factors, such as lactoperoxidase, lactoferrin, and immunoglobulin, all with the capability to kill or to inhibit a large spectrum of pathogens (Zhang et al., 2008; La Torre et al., 2010; Nazzaro et al., 2010). Lysozyme antibacterial activity is due to its capacity to catalyze the hydrolysis of the $\beta(1-4)$ glycosidic links between *N*-acetylmuramic acid and *N*-acetylglucosamine in the bacterial cell wall polysaccharides, working in synergy with lactoferrin and immunoglobulins (Marseglia et al., 2013). Donkey lysozyme is of the c-type, which is 129 AA long, and exhibits 50% homology to the human protein (Callewaert and Michiels, 2010). The lysozyme content in jenny milk ranges between 1.0 and 3.7 mg/mL, according to the lactation stage and the production season (Zhang et al., 2008; Galassi et al., 2012; Vincenzetti et al., 2012), and is much higher than in cow (0.13 μ g/mL), ewe (0.20 μ g/mL), or goat milk (0.25 μ g/mL; Fratini et al., 2006; Scharfen et al., 2007). Furthermore, previous studies (Cosentino and Paolino, 2012; Cosentino et al., 2013) showed that the addition of jenny milk in ewe milk led to the inhibition of co-

liform bacteria in the experimental cheese group, but the treatment did not significantly affect the number of *Clostridium butyricum* spores (Cosentino and Paolino, 2012; Cosentino et al., 2013). The lower content of coliforms in treated ewe cheese was in agreement with results from the literature on reduced growth of *C. butyricum* in Grana Padano PDO (Iaconelli et al., 2008; Dragoni et al., 2011) and Gouda cheese (Bester and Lombard, 1990). Martínez-Cuesta et al. (2010) observed a higher contamination of *Clostridia* in Manchego control cheese compared with that treated with lysozyme hen egg white.

In the current paper, the effect of jenny milk as an inhibitor of blowing defects was evaluated. In particular, it was verified whether increasing additions of jenny milk to pasteurized cow milk reduce the late blowing defects in semihard cheese. Moreover, the acceptability of cheeses was evaluated through a consumer test.

MATERIALS AND METHODS

Animals

Bulk cow milk and jenny milk were collected from 2 farms, both situated in Basilicata region (southern Italy) at an altitude of about 700 m above sea level. Cow milk samples were collected from pluriparous Holstein Friesian cows, weighting 650 ± 10 kg, and fed a triticale silage feed composed of triticale silage (50.25%), alfalfa hay (6.78%), maize meal (8.79%), soybean meal (8.04%), beet pressed pulp (1.51%), maize gluten meal (1.51%), hay vetch and oats (3.77%), flaked maize (6.78%), cotton seed (2.51%), vitamin-mineral supplement (1.27%), and water (8.79%).

Jenny milk samples were collected from a local population of pluriparous jennies, aged between 7 and 10 yr, and fed on a diet consisting of ad libitum oat hay and an integration of 1 kg of concentrate, characterized by the following mixture: flaked corn (37%), oats (30%), locust bean crushed (9%), wheat bran (8%), dehydrated alfalfa (8%), beet pulp dried (6%), and vitamin-mineral supplement (2%).

Milk Sample Analysis

Bulk cow and jenny milk were collected the same day from both farms by a mechanical milking apparatus. After milk collection, samples were immediately refrigerated at 4°C and transported to the laboratory for analytical determinations.

On raw cow and jenny milk, we measured pH (HI931410, Hanna Instruments, Padova, Italy), protein, fat, and lactose content according to the International

Table 1. Parameters of raw milk

Parameter	Milk	
	Cow	Jenny
Protein, %	3.30	1.70
Fat, %	3.70	1.30
Lactose, %	4.10	6.40
DM, %	11.70	9.60
Ash, %	0.50	0.40
Lysozyme, mg/mL	0.18	2.03
pH at 25°C	6.70	7.10
<i>Clostridium tyrobutyricum</i> , log ₁₀ cells/mL	230	<10
Total bacterial count, log ₁₀ cfu/mL	4.40	2.90

Dairy Federation standard (ISO, 2013a), as well as DM and ash content (AOAC, 1990). Lysozyme content was determined by HPLC (Agilent Technologies, Palo Alto, CA) according to the method described by Pellegrino and Tirelli (2000). Plate count agar was used for enumeration of total bacterial viable count (ISO, 2013b), whereas the most probable number method was used to estimate the number of *C. tyrobutyricum* cells. Cow and jenny raw milk characteristics are reported in Table 1.

Clostridium tyrobutyricum

To increase the probability of inducing late blowing, we used a high dose of spores, 3 log cfu/mL of milk, derived from cultures of *C. tyrobutyricum* CLST01, as a cheese blowing agent. Spores were obtained following inoculation in reinforced clostridial medium (RCM; Oxoid, Basingstoke, UK) in anaerobic conditions at 37°C for 5 d. After centrifugation ($5000 \times g$, 15 min, 20°C), the pellet was washed twice with sterile distilled water, resuspended in reconstituted skim milk, and heat-shocked at 80°C for 20 min to kill off vegetative cells before cheese manufacture. Spore counts were determined on RCM agar (1.5%, wt/vol) after anaerobic incubation at 37°C for 3 d.

The strain was maintained as frozen stocks in reconstituted 11% (wt/vol) skim milk containing 0.1% (wt/vol) ascorbic acid in the culture collection of School of Agriculture, Forestry, Food and Environmental Sciences, University of Basilicata, and routinely propagated (1%, vol/vol) in RCM for 72 h at 37°C under anaerobic conditions.

Semihard Cheese Manufacture

Pasteurized cow milk was first heat-treated at 65°C for 30 min, and then cooled to 37°C. Thereafter, a fixed amount (40 L) of milk was poured into 8 vats, to which different aliquots of raw jenny milk were added (Table

Table 2. Experimental design of semihard cheese manufacture

Item	Group A ¹				Group B ²			
	A1	A2	A3	A4	B1	B2	B3	B4
Cow milk, L	40	40	40	40	40	40	40	40
Jenny milk, L	—	0.8	1.6	3.2	—	0.8	1.6	3.2
<i>Clostridium tyrobutyricum</i> , log ₁₀ cfu/mL	—	—	—	—	3	3	3	3

¹A1, A2, A3, and A4 are the volumes of cow and jenny milk used for the manufacture of A (uncontaminated) group semihard cheeses.

²B1, B2, B3, and B4 are the volumes of cow milk, jenny milk, and *Clostridium tyrobutyricum* spores used for the manufacture of B (contaminated) group semihard cheeses.

2). To each vat, 0.2 g/L of kid rennet (activity 1:10,000; Caglio Camoscio CSC 95/75, DMS Segrate, Italy) was then added.

The vats were then split into 2 groups (A and B). The milk contained in 4 vats (group B: B1, B2, B3, and B4) was deliberately contaminated with approximately 3 log₁₀ cfu/mL milk of *C. tyrobutyricum* CLST01 to induce butyric acid fermentation and consequent blowing defect (Gómez-Torres et al., 2014). No clostridia spores were added to the milk contained in other 4 vats (group A: A1, A2, A3, and A4).

After 50 min, the curds of each vat were cut into particles of 0.5 cm diameter. All curds were then pressed into cylindrical molds (diameter = 14 cm, height = 8 cm). Sixteen cheeses weighing about 1 kg were obtained from each cheese group. After 24 h of draining, cheeses were salted in containers with sterile brine (200 g/L of NaCl, pH 5.40) for 2 h and then stored at 20°C for 4 d. Finally, cheeses were left in a ripening room (13–15°C, air humidity = 80–85%) for 120 d. The whole experiment was repeated twice.

Detection of LBD

During ripening, the occurrence of external blowing defects was observed monthly on a total of 64 cheeses (4 cheeses × 4 vats × 2 cheese groups × 2 experiments) by visual inspections according to Matijašić et al. (2007). For each group, the incidence of defects during seasoning was calculated as the number of cheeses showing the defects divided by 8 (total number of cheeses per vat). At the end of ripening, appearance of irregular holes, cracks, and splits within the cheese matrix was acquired by a digital camera and images were used in an explicative scheme (Figure 1).

Physical Parameters of Cheeses

At the end of the ripening, both group cheeses were weighed and the diameter was recorded. Then, cheeses were cut into 3 cross sections (a, b, c; Figure 1), whose heights (h1, h2, h3) were also recorded.

Cheese Analysis

Both group cheeses were sampled according to IDF (1995) procedure to perform the following analyses: pH (pH-meter HI931410, Hanna Instruments, Padua, Italy); water activity (a_w) according to ISO (2001); and lysozyme content (Pellegrino and Tirelli, 2000). Total bacterial viable count and the number of *C. tyrobutyricum* spores were also assessed in both group cheeses.

Consumer Evaluation

The acceptability of semihard cheeses from group A was assessed by 80 regular consumers of cheese, equally distributed by age and sex. A small amount (20 g) of each type of cheese (A1, A2, A3, and A4) was coded with 3-digit random numbers and offered to consumers. They were asked to evaluate color, odor, flavor, and texture using a 9-point liking scale. The consumer test was performed in sensory booths (UNI-ISO, 1990) provided with a computer system for data acquisition (Software FIZZ ver. 1.3.1, Biosystèmes, Couternon, France).

Statistical Analysis

Within each cheese group, physical, chemical, microbiological, and acceptability data were subjected to ANOVA, and means were compared by least significant difference. Differences at $P < 0.05$ were considered significant. All statistical analyses were performed using R (R Development Core Team, 2010).

RESULTS AND DISCUSSION

Detection of LBD

After 30 d of ripening (Table 3), no sign of LBD was observed in group A. Two months later, defects were observed only in A1, with 62.50% (5 from 8) of cheeses being slightly blown. After 90 d, signs of defects occurred in A1 (87.50% of cheeses were blown) and in A2 (75% of cheeses were slightly blown), whereas A3

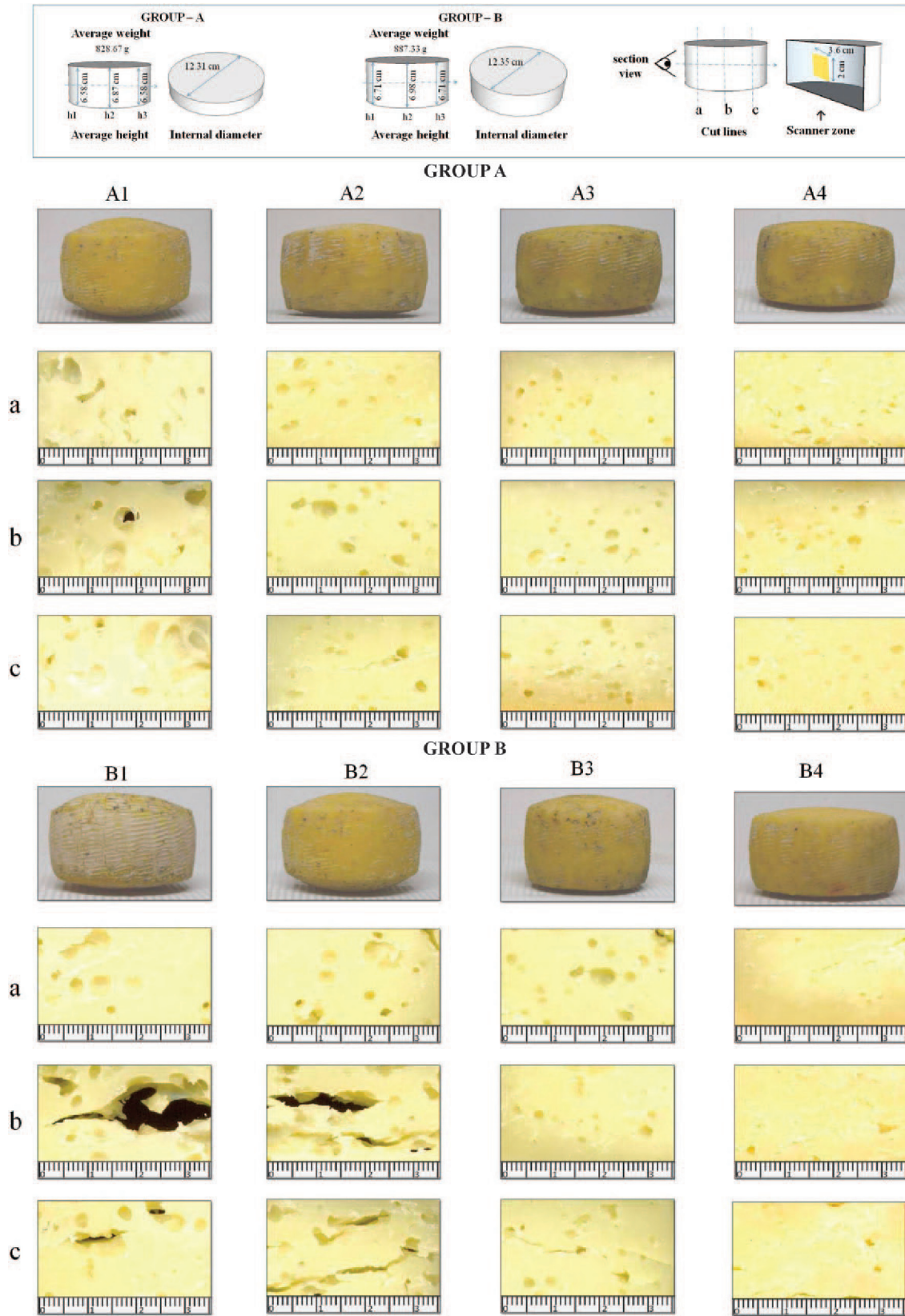


Figure 1. Signs of late blowing defects in both cheese groups after 120 d of ripening; h1, h2, and h3 are the respective heights of cross sections 1, 2, and 3. Color version available online.

and A4 showed no defects. Similarly, defects occurred in both A1 and A2 at the end of ripening (120 d), although the incidence of defects increased. Concerning group B, after 30 and 60 d of ripening, 62.50% of B1 and B2 cheeses showed signs of defects, whereas no signs were detected in B3 and B4. After 90 d, defects were observed not only in B1 and B2 (the incidence of blown and cracked cheeses was 87.50%), but also in B3, with 75% of cheeses appearing slightly blown. At the end of ripening, the previously mentioned defects occurred in B1, B2, and B3 at a higher rate.

The differences in visual signs of LBD at the end of ripening were reflected in differences in physical parameters. Within the A group, the height of the 3 cross sections (h1, h2, and h3) were significantly ($P < 0.001$) lower in A3 and A4 compared with the other cheeses (Table 4). Significant differences ($P < 0.001$) in cross section height (h1, h2, and h3) were also detected in the B group, with B4 showing the lowest height value (Table 5).

The appearance of the 3 cross sections of both cheese groups at the end of ripening is shown in Figure 1. As can be seen, both A1 and A2 were blown outside and characterized by many irregular holes inside, albeit they were smaller in A2. Instead, A3 and A4 showed a more uniform texture, as the holes were smaller. With regard to the treated group, B1 was the most blown outside, followed by B2. Both had large irregular holes inside with large splits in the central cross section. From B3 to B4, defects were less evident as consequence of the increasing addition of jenny milk during cheese making. Overall, these results seem to indicate that the addition of increasing amounts of jenny milk determines a reduction of LBD during ripening.

Analysis of Cheese

The results of chemical and microbiological analysis for A and B cheeses at the end of ripening are shown in Tables 4 and 5, respectively. The effect of jenny milk addition was significant on several parameters. Within each group, the pH value was significantly ($P < 0.001$) lower in cheeses made with the highest aliquot of jenny milk (A4 and B4) than in the other cheeses. According to Galassi et al. (2012), the addition of jenny milk during cheese making led to a decrease in pH, which, in turn, promotes lactic fermentation. The high values of pH observed in cheeses made with lower aliquots of jenny milk may be due to deacidification resulting from metabolic activity of *C. tyrobutyricum* (Matijašić et al., 2007).

Water activity is an index of the free water that can support the growth of particular groups of bacteria, yeast, and mold (Hickey et al., 2013). Generally, low a_w

Table 3. Signs of blowing defects in A (uncontaminated) and B (contaminated) group cheeses during ripening

Days of ripening	Late blowing defects	Cheese groups ¹							
		A1	A2	A3	A4	B1	B2	B3	B4
30	Signs	ND ²	ND	ND	ND	Slightly blown	Slightly blown	ND	ND
60	Appearance ³	100%	100%	100%	100%	62.50%	62.50%	100%	100%
	Signs	Slightly blown	ND	ND	ND	Blown, cracked	Blown	ND	ND
90	Appearance	62.50%	100%	100%	100%	62.50%	62.50%	100%	100%
	Signs	Blown	Slightly blown	ND	ND	Blown, cracked	Blown, cracked	Slightly blown	ND
120	Appearance	87.50%	75%	100%	100%	87.50%	87.50%	75%	100%
	Signs	Blown	Slightly blown	ND	ND	Blown, cracked	Blown, cracked	Slightly blown	ND
	Appearance	100%	100%	100%	100%	100%	100%	100%	100%

¹A1, A2, A3, and A4 are semihard cheese samples made by adding, respectively, 0, 0.8, 1.6, and 3.2 L of jenny milk to a fixed volume (40 L) of cow milk. B1, B2, B3, and B4 are semihard cheese samples made by adding, respectively, 0, 0.8, 1.6, and 3.2 L of jenny milk to a fixed volume (40 L) of cow milk contaminated with *Clostridium tyrobutyricum* spores.

²Not detected.

³Appearance is the incidence of signs of late blowing defects detected in semihard cheese samples during ripening. It was calculated as the number of cheeses showing the signs divided by 8 (total number of cheeses per vat).

Table 4. Physical, chemical, and microbiological parameters of group A (uncontaminated) cheeses

Cheese ¹	Cross section height, ² cm			pH at 25°C	Water activity	Lysozyme, mg/kg	<i>Clostridium tyrobutyricum</i> , log ₁₀ cfu/g	Total bacterial count, log ₁₀ cfu/g
	h1	h2	h3					
A1	7.10 ^a	7.54 ^a	7.31 ^a	5.14 ^a	0.87 ^a	0.80 ^a	93.3 ^a	6.77 ^a
A2	6.94 ^a	7.37 ^a	7.16 ^a	5.34 ^b	0.91 ^b	0.93 ^b	70.00 ^b	6.63 ^b
A3	6.29 ^b	6.38 ^b	6.04 ^b	5.21 ^a	0.90 ^b	1.05 ^b	38.33 ^c	6.59 ^c
A4	6.00 ^b	6.24 ^b	5.80 ^b	4.81 ^c	0.90 ^b	1.57 ^c	30.00 ^d	6.55 ^d

^{a-d}Means within each column with different superscripts differ significantly ($P < 0.05$).

¹A1, A2, A3, and A4 are semihard cheese samples made by adding, respectively, 0, 0.8, 1.6, and 3.2 L of jenny milk to a fixed volume (40 L) of cow milk.

²h1, h2, and h3 are the respective heights of cross sections 1, 2, and 3 (Figure 1).

values inhibit microbial growth. According to Beuchat (1981), for most foodstuffs an a_w below 0.6 provides an effective control of microbial growth. In the present study, the value of a_w observed in both cheese groups (0.8–0.9) was consistent with that reported in literature for this kind of food (Beuchat, 1981). However, significant differences ($P < 0.001$) were noted within each group; the value of a_w was significantly lower in A1 than in other cheeses. Similarly, among the B cheeses, the lowest value of a_w was observed in B1.

Concerning the lysozyme content, the value was significantly ($P < 0.001$) the highest in A4 and in B4 (1.57 and 1.52 mg/kg, respectively). This result seems to be related to the increasing addition of jenny milk to cow milk during cheesemaking. The observed values are consistent with previous studies, in which the content of lysozyme in cheese was found to range from 50 to 350 $\mu\text{g/g}$ of cheese (Pellegrino and Tirelli, 2000), with a maximum of 400 $\mu\text{g/g}$ of cheese (Ávila et al., 2014).

Regarding the microbiological analysis, the counts of *C. tyrobutyricum* spores were significantly higher ($P < 0.001$) in A1 (93.3 log₁₀ cfu/g) compared with other cheeses (A2 = 70.0 log₁₀ cfu/g; A3 = 38.33 log₁₀ cfu/g; A4 = 30.0 log₁₀ cfu/g). Similarly, the number of *C. tyrobutyricum* spores was significantly higher ($P < 0.001$) in B1 blown cheeses (129.0 log₁₀ cfu/g) compared with the other cheeses (B2 = 97.7 log₁₀ cfu/g; B3 = 66.83 log₁₀ cfu/g; B4 = 61.5 log₁₀ cfu/g). Total bacteria count

was also significantly lower ($P < 0.001$) in A4 (6.55 log₁₀ cfu/g) than in other cheeses. The same trend was observed in B cheeses, with B4 showing the lowest value (7.53 log₁₀ cfu/g).

These results confirm that lysozyme has an important antimicrobial activity, as it is capable of killing or inhibiting a broad spectrum of pathogens (Chiavari et al., 2005; Polidori and Vincenzetti, 2007; Salerno et al., 2011). As reported by Ávila et al. (2014), the growth of *C. tyrobutyricum* strains, which are responsible for LBD, can be inhibited by this enzyme. Martínez-Cuesta et al. (2010) reported that the addition of 25 $\mu\text{g/mL}$ of lysozyme to milk can prevent LBD caused by *C. tyrobutyricum* in semihard cheese. The antibacterial activity of milk lysozyme is also well established (Benkerroum, 2008). According to Galassi et al. (2012), jenny milk is an effective substitute for egg lysozyme in Grana Padano cheesemaking. Galassi et al. (2012) found that the addition of 10 L of jenny milk to 500 L of cow milk improves significantly several physicochemical and microbiological aspects of cheese. Cosentino et al. (2013) also reported that the addition of jenny milk in ewe milk can inhibit coliform bacteria and prevent late blowing in cheese.

Our results seem to indicate that the antimicrobial activity of milk lysozyme increased with its concentration in cheese. Because of the increasing addition of jenny milk, lysozyme content increased from A1 to A4

Table 5. Physical, chemical, and microbiological parameters of group B (contaminated) cheeses

Cheese ¹	Cross section height, ² cm			pH at 25°C	Water activity	Lysozyme, mg/kg	<i>Clostridium tyrobutyricum</i> , log ₁₀ cfu/g	Total bacterial count, log ₁₀ cfu/g
	h1	h2	h3					
B1	7.22 ^a	7.57 ^a	7.35 ^a	5.14 ^a	0.84 ^a	0.80 ^a	129.00 ^a	7.79 ^a
B2	7.04 ^a	7.41 ^a	7.24 ^a	5.33 ^b	0.92 ^b	0.93 ^b	97.70 ^b	7.63 ^b
B3	6.59 ^b	6.75 ^b	6.52 ^b	5.25 ^c	0.89 ^c	1.00 ^b	66.83 ^c	7.58 ^c
B4	6.01 ^c	6.52 ^c	5.71 ^c	4.95 ^d	0.90 ^{bc}	1.52 ^c	61.50 ^d	7.53 ^d

^{a-d}Means within each column with different superscripts differ significantly ($P < 0.05$).

¹B1, B2, B3, and B4 are semihard cheese samples made by adding, respectively, 0, 0.8, 1.6, and 3.2 L of jenny milk to a fixed volume (40 L) of cow milk contaminated with *C. tyrobutyricum* spores.

²h1, h2, and h3 are the respective heights of cross sections 1, 2, and 3 (Figure 1).

and from B1 to B4. This increase in lysozyme content resulted in a progressive decrease in the number of *C. tyrobutyricum* spores and total bacterial count. These results are probably explained by the fact that lysozyme lyses the cell wall of certain gram-positive bacteria by splitting $\beta(1-4)$ linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan, the components making up bacterial cell walls (Mine et al., 2004). This important antimicrobial activity led to the inhibition of spore-forming clostridia strains and, consequently, to a reduction of the occurrence of LBD in cheese, as confirmed by chemical and physical parameters as well as by visual observations of the appearance of cheeses.

Consumer Evaluation

As shown in Table 6, the mean liking scores of group A cheeses were well above the central point (5 = neither pleasant nor unpleasant) of the liking scale for all sensory parameters (i.e., color, odor, flavor, and texture), thus indicating that the products were characterized by a good eating quality. Moreover, the ANOVA showed no significant differences among cheeses for each sensory parameter. This result is in line with that of Galassi et al. (2012), and indicates that the addition of jenny milk in cheese making does not affect the acceptability of the products.

General Discussion

According to Vissers et al. (2007b), 3 main options exist for preventing LBD: (1) minimizing butyric acid bacteria (BAB) spore concentrations in raw milk; (2) removing BAB spores from raw milk via bacto-fugation; and (3) preventing the growth of BAB in cheese by adding inhibitory agents (e.g., lysozyme and nitrate) to cheese milk. The effectiveness of the latter option was verified in the present study by adding different

aliquots of jenny milk, which is characterized by a high content of lysozyme, to cow milk during cheese making.

Strengthening a previous study on cheese made with ewe milk (Cosentino et al., 2013), our results confirm the important antimicrobial activity of jenny milk lysozyme, as it was also effective in controlling the occurrence of LBD in semihard cheese. In particular, the addition of jenny milk at a concentration of 3.85% was sufficient to prevent the occurrence of signs of LBD in A3 cheese until the end of ripening. This result was probably related to a higher content of lysozyme found in A3 cheese compared with A1 (control) cheese, which, in turn, played a key role in preventing the growth of *C. tyrobutyricum* spores. The results concerning B group cheeses seem to further confirm this important inhibitory activity of jenny milk lysozyme; in fact, the addition of jenny milk at a concentration of 7.41% to cow milk deliberately contaminated with *C. tyrobutyricum* spores prevented the occurrence of visual signs of LBD in B4 cheese. Moreover, the addition of jenny milk to cow milk during cheesemaking, at least to a maximum concentration of 7.41%, does not affect semihard cheese acceptability, and no significant differences were observed among cheeses according to consumers concerning sensorial aspects.

In conclusion, we strongly recommend jenny milk lysozyme as a viable alternative to egg lysozyme for controlling LBD in cheese. The use of milk lysozyme could be successfully exploited in niche productions of cheese by the addition of jenny milk during cheesemaking. This may also have positive implications for existing donkey farms, as well as for the conservation or reintroduction of this species in marginal areas (Cosentino et al., 2015). Finally, further studies are needed to assess the usefulness of milk lysozyme for controlling the growth of deleterious microorganisms, such as *Bacillus* and *Listeria*, and hence prolonging the shelf life of fresh fruits and vegetables, seafood, meats and sausages, as well as cheeses.

Table 6. Consumer liking scores of group A (uncontaminated) cheeses

Cheese ¹	Sensory parameter ²			
	Color	Odor	Flavor	Texture
A1	6.48	6.28	6.34	6.34
A2	6.26	5.97	6.19	6.26
A3	6.10	5.57	5.62	5.83
A4	6.28	5.74	5.91	5.86

¹A1, A2, A3, and A4 are semihard cheese samples made by adding, respectively, 0, 0.8, 1.6, and 3.2 L of jenny milk to a fixed volume (40 L) of cow milk.

²The sensory parameters of uncontaminated cheeses were evaluated by 80 consumers through a 9-point liking scale. Means within each column did not differ significantly.

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