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Efficient milking hygiene reduces bacterial spore contamination in milk

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Clostridia in the milk can lead to late blowing, a cheese defect. Clostridia are ubiquitous, deriving from both the farm environment and the feed ingested by the cows, and are transferred into the milk through faecal contamination. Our aim was to investigate the effect of different in-parlour practices on the content of anaerobic spore-forming bacteria in milk, and to monitor the variation in spore content in the feed and environment. The experiment, conducted in an experimental dairy during autumn, was repeated in exactly the same way for two consecutive years. The experimental design applied three different milking routines in three consecutive 7-d periods: forestripping alone (F); forestripping and post-dipping (F+Post); pre-dipping, wiping, forestripping and post-dipping (Pre+F+Post). Teat skin swabs and samples of feed, faeces, bedding materials and milk were collected for microbiological analyses. The dietary forage of the lactating cows included maize silage, which, in both years, was found to have the highest level of clostridial spore contamination. Pre-dipping with a detergent/emollient solution, and drying with a disposable paper towel, proved much more efficient in reducing spore contamination than forestripping alone, both on the teats (1·30 *vs.* 2·20 $\log_{10}$ MPN/swab; $P < 0{\cdot}001$) and in the milk (1·82 *vs.* 2·47 $\log_{10}$ MPN/L, $P < 0{\cdot}02$), while post-dipping had little influence on spore count. The standard plate count in milk was significantly lower with Pre+F+Post treatment than with F (3·80 *vs.* 4·51 $\log_{10}$ CFU/mL, $P < 0{\cdot}01$). The teat preparation procedure did not influence the lactic acid bacterial levels in the milk, which is very positive in that decreased lactic acid bacterial content can lessen raw milk cheese quality.

Keywords: Milk, *Clostridium*, spores, milking, propionibacteria.

Anaerobic spore-forming bacteria (ASFB) are important spoilage organisms in a variety of dairy foods. Indeed, the anaerobic interior in semi-hard and hard cheeses provides a favorable environment for some *Clostridium* species that metabolise lactate into hydrogen gas, resulting in 'late blowing' defects (Miller et al. 2015). Gas defects in cheese may be caused by *Clostridium tyrobutyricum*, *C. beijerinckii*, *C. butyricum*, and also by *C. sporogenes*, which can produce gas through proteolysis (Doyle et al. 2015).

Late blowing is seen as cracks and slits in the cheese paste associated with abnormal aroma and flavor. It is a defect that can be found in some types of European cow milk cheeses, many of them made with unpasteurised cow milk, such as Grana Padano, Parmigiano Reggiano, Edam, Saint-Nectaire, Gouda, Emmental, Gruyer, Comte and sheep milk cheese Machengo (Gómez Torres et al. 2015). In Grana Padano production, late blowing causes a notable loss in cheese production, affecting from 15 to 35% of cheese wheels according to Borreani & Tabacco (2008). Conversely Garde et al. (2011), in a study on Manchego cheese made with ovine milk, reported that 0·63% of the total summer cheese production was affected by late blowing, despite the fact that 91% of the milk samples showed an MPN spore count above the level recommended to avoid late blowing defect in ovine milk cheese.

A recent study on Grana Padano by Feligini et al. (2014) showed a highly significant effect of season on the *Clostridium* spp. population: during winter *C. butyricum* and *C. sporogenes* prevailed in curd and milk respectively; during spring the clostridial population was very closely associated with *C. tyrobutyricum,* while in summer and autumn *C. beijerinckii* dominated. According to the authors of this study, the species distribution over the

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seasons suggests that the blowing defect in Grana Padano might not be due to the metabolic activity of a single species, but could depend on the synergistic action of several spore-formers transferred into the milk.

For milk, the most important sources of spore contamination are soil, feed (especially ensiled forages), bedding materials and faeces (Zucali et al. 2015). The transfer of spores from soil to crops during cropping and harvesting results in spore contamination of silage. Moreover, spore concentrations can increase during storage in the silo, this depending on the conditions prevailing in the silage and the requirements of the microorganisms (Driehuis, 2013). Spores ingested by the animals pass through the digestive tract and are excreted with the faeces, causing contamination of bedding materials and teat skin, with subsequent possible entry into the milk at milking time. Indeed, bedding material can act as a reservoir for ASFB, as shown by Magnusson et al. (2006).

Vissers et al. (2007a) studied the transmission of microorganisms to milk *via* dirt (i.e., faeces, bedding material, soil, or their combination) attached to the exterior of the teats. They concluded that although spore concentration in faeces is the major factor affecting the butyric acid bacteria spore concentration in bulk tank milk, teat hygiene could considerably reduce the risk of contamination. In a recent review Doyle et al. (2015) concluded that good quality silage, stringent cleaning routines of shed/cubicle and of parlour/milking equipment, as well as rigorous udder cleaning and teat preparation prior to milking, are all necessary measures to reduce the risk of bulk tank milk contamination by ASFB.

Even though spores are frequently associated with inadequate cow hygiene, the role of udder cleanliness and milking routine in connection with spore contamination of milk has been the subject of only a limited number of studies, In one of these, Zucali et al. (2015) found that the presence, in the herd, of more than 40% of lactating cows with dirty udders increased the average spore contamination of the milk by 15%. Nadeau et al. (2010) observed that Swedish herds with poor cow cleanliness had higher milk spore counts. Considering the milking routine, Melin et al. (2002) showed that good teat cleaning before milking could lead to substantial spore contamination control. Magnusson et al. (2006) showed that the most effective method to reduce spore content in milk was the use of a moist towel (washable) followed by drying with a paper towel. Doyle et al. (2015) reported that milking parlour practices, such as a 20 s dipping/cleansing of teats using individual paper or cotton towels, reduced the bacterial load on a cow's teats and subsequently the ASFB in the milk.

In their conclusions, Miller et al. (2015) underlined that future research should focus on the importance of bedding material and milking routine to elucidate the role that these factors play in spore transfer from the environment to milk. Also the teat skin is a source of bacteria useful for cheesemaking, such as lactic acid bacteria (LAB) and propionibacteria, and their levels could be affected by milking hygiene and disinfection practices (Vacheyrou et al. 2011).

The main purpose of the study was to investigate the effect, on ASFB count in milk, of different in-parlour teat preparation practices (forestripping, pre-dipping and post-dipping). Moreover, the experiment was aimed at studying the effect of milking operations on a number of other microbiological parameters of milk, and to monitor the variation of ASFB along the entire contamination chain: feeds, faeces, bedding materials and teat skin.

Materials and methods

Experimental design

The trial was conducted at the experimental farm of the University of Milan located in Landriano (Pavia, Northern Italy), in the Po plain. The herd was composed of 80 Italian Friesian lactating cows. Lactating animals were housed in loose housing cubicle systems without access to pasture or outside yards and milked twice a day in a herringbone 7 + 7 milking parlour. The cubicle bedding was covered with rubber mats and chopped straw, renewed weekly.

The experiment was repeated in two consecutive years (2013 and 2014) in the same season (autumn), with the same design, and with the same dairy farm staff. The experimental design included the application of three different milking routines in three consecutive 7-d periods:

F = forestripping alone
F + Post = forestripping + post − milking teat dip
Pre + F + Post = pre − milking teat dip + wiping + *forestripping* + *post* − *milking* teat dip.

During each experimental period, the milking routines were applied on both daily milkings (2 milkings a day for 7 d).

Commercial products were used for the pre and post-milking teat dip: the pre-dipping solution (Predipping foam, Deavit, Milan, Italy) included only detergent and emollient agents, while the post-dipping product (ADVANCE G3, IRCA Service s.p.a. Fornovo San Giovanni – BG, Italy) included glycolic acid. After applying the pre-dipping product, the teats were dried (wiping) with individual paper towels before the forestripping and the attachment of the milking cups.

Once a day the cows were fed a total mixed ration (TMR); the composition and chemical analysis of the rations in the two years are shown in Table 1.

Cow and cubicle cleanliness assessment

During the experimental periods, dairy cow hygiene scores were assessed by direct observation in the milking parlour. In accordance with Schreiner & Ruegg (2003), the udder, flanks and legs of each cow were evaluated on a 4-point scale system, where a score of 1 indicates very clean skin

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Table 1. Ingredients (% DM), chemical composition (% DM unless otherwise stated) and clostridial spore content (log_{10} MPN/g) of the total mixed rations (TMR) fed to lactating cows in the two years of experiment

	Ingredients (% DM)		Clostridial spore content (log_{10} MPN/g)	
	1st year	2nd year	1st year	2nd year
Maize silage	28·4	26·4	5·38	2·97
High moisture ear maize silage		9·24		1·56
Lucerne silage	9·46		2·58	
Wheat and pea silage		9·46		0·56
Lucerne hay	6·29	6·79	1·97	2·32
Italian ryegrass hay	5·21		2·38	
Concentrate mix	50·6	48·1		
TMR			5·67	4·18
DM (% as fed)	50·2	49·5		
Ash	8·18	7·30		
CP	16·4	15·7		
EE	3·34	2·90		
NDF	36·3	30·0		
ADF	21·9	21·2		
ADL	4·60	4·90		

and 4 a skin completely covered with dirt. The percentage of animals with scores of 3 and 4 for udder, flanks and legs was calculated.

Cubicle cleanliness was assessed according to a 3-point scheme on a random sample of cubicles, assuming score 1 for clean bedding material, 3 for very dirty bedding material.

Sampling

In the last three days of each experimental period, samples of feed (TMR and forage, $n = 1$), faeces ($n = 2$), bedding material ($n = 2$), teat swabs ($n = 10$) and milk ($n = 3$) were collected for microbiological analyses. TMR and forage samples were collected in the morning during ration preparation. Maize silage samples were taken from both the core and peripheral areas of the face of the bunker silo. In year two, additional TMR samples were collected every hour eight times after the first morning distribution, and analysed for ASFB contamination to study the spore variation during the period in which the TMR remains in the manger. Faeces samples were collected during milking; a pooled sample of fresh material was obtained by taking equivalent faeces amounts from 10 random cows. The samples of bedding material were taken randomly from 5 different cubicles and then pooled. Moistened commercial paper towels, with no disinfectant agent, were used as swabs to wipe the whole teat surface after the pre-milking cleaning and before claw attachment, as described by Zucali et al. (2011). During milking, swabs were taken on a single teat of 10 cows selected randomly from the herd. The teat swabs were immediately placed in sterile plastic 'zip-loc' bags. The bulk milk was mixed in the tank after the morning milking, and samples were collected with a probe. All the samples were transported to the laboratory under refrigeration (4 °C) no later than 12 h after collection, and submitted to microbiological analyses.

Feed chemical analyses

The TMR samples were analysed for chemical composition as follows: dry matter (DM) was determined following the AOAC procedure (AOAC, method 945·15, 1995), and organic matter was calculated as weight lost upon ignition at 600 °C (AOAC, method 942·05, 1995). The crude protein (CP) content was determined by the macro-Kjeldahl technique (AOAC, method 984·13, 1995) using a 2300 Kjeltec Analyzer Unit (FOSS, Hillerød, Denmark). Ether extract was determined following the method 920·29 of the AOAC (1995). Neutral detergent fibre (NDF) was determined according to Mertens (2002), with the addition of sodium sulphite and α-amylase to the neutral detergent solution. Acid detergent fibre (ADF), determined not sequentially to NDF, and acid detergent lignin (ADL) were calculated according to the method of Van Soest et al. (1991) using the Ankom 200 fibre apparatus (ANKOM Technology Corporation, Fairport, NY). Fibre fractions are reported on an ash-free basis.

Microbiological analyses

Feed, faeces, bedding material, teat swabs and milk were subjected to microbiological analysis to monitor the changes in ASFB, bacterial count and propionibacteria. In addition, coliform and lactic acid bacteria in the milk were evaluated. All the samples were transported to the laboratory under refrigeration (4 °C) and analysed the morning after collection within 12 h.

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For the microbiological analysis, the forage samples were chopped for 1 min in a sterile homogeniser, then (10 g) were suspended in a 1 : 10 PSS (peptone salt solution, 1 g of bacteriological peptone and 9 g of sodium chloride per litre), and homogenised twice for 2 min at maximum speed using a Stomacher blender (BagMixer 400, Interscience). Ten grams of faeces were suspended in a 1 : 10 PSS and homogenised twice for 1 min at maximum speed using a Stomacher. For the teat swabs, PSS was added to every bag and samples were homogenised at high speed for 30 s in a Stomacher blender. All the homogenates were serially diluted in quarter-strength Ringer solution.

The anaerobic spore content was obtained through the Most Probable Number (MPN) performed with three 10-fold dilutions with three tubes at each dilution, as reported by Zucali et al. (2015). In order to determine the *Clostridium* species in faeces, bedding material, teat swabs and milk (*C. tyrobutyricum*, *C. butyricum*, *C. beijerinckii*, and *C. sporogenes*) positive tubes were analysed by multiplex PCR according to Morandi et al. (2015). Standard plate count (SPC) and coliform count (CC) were determined using Petrifilm (3M, St. Paul, MN, USA), and the plates were incubated at 30 °C for 72 and 24 h, respectively. LAB were determined on de Man Rogosa and Sharpe (MRS) agar (Biolife, Milan, Italy); the plates were incubated anaerobically (Anaerocult A, Merck Millipore, Darmstadt, Germany) at 30 °C for 72 h. P2 agar (peptone 5 g; beef extract, 3 g; yeast extract, 5 g; sodium lactate, 1 g; agar 15 g per litre) incubated anaerobically at 30 °C for 7 d was used for the enumeration of the propionibacteria.

Statistical analysis

Data on microbiological contamination and hygiene score were analysed by Proc GLM (SAS, 2009), using the following model:

$$Y_{ijk} = \mu + T_i + Y_j + TY_{ij} + e_{ijk}$$

where Y_{ijk} are the dependent variables, μ the general mean, T_i the effect of milking routine ($i = 1$–3; 1, Forestripping; 2, forestripping+post-dipping; 3, pre-dipping+forestripping+post-dipping), Y_j is the effect of the year of the trial ($j = 1$–2; 2013–2014), TY_{ij} is the interaction between treatment and year, and e_{ijk} is the residual error. Data are reported as least squares means of the two years.

Results and discussion

In the two years of the experiment, the total mixed ration (TMR) fed to the lactating cows was characterised by an average forage content of approximately 50% dry matter (DM). The average percentage of maize silage of the total ration DM was high (27·4%), in agreement with the average maize silage inclusion in TMR fed to lactating cows in Pirondini et al. (2012). Of the feed included in the TMR, the maize silage had the highest spore content in both years of the experiment (Table 1). As recently demonstrated by some studies, maize silage has been shown to have a high spore content (Vissers et al. 2007b; Zucali et al. 2015). The spore content of the maize silage in the first year was much higher (5·38 $\log_{10}$ MPN/g) than in the second (2·97 $\log_{10}$ MPN/g), and both values are higher than those reported by other authors for maize silage from the Po plain (Colombari et al. 2001; Borreani & Tabacco, 2008). The spore content of TMR was very high in both years and higher than the single forage spore contents (Table 1). This agrees with the results of Vissers et al. (2007b) who found that the mixed silage in the barn had a higher spore content than the individual forage components.

For an in-depth analysis of how ASFB contaminates TMR, a short experiment was conducted in year two to investigate the possible growth of anaerobic spore forming bacteria (ASFB) during the period TMR remains in the manger. TMR samples were collected at hourly intervals on eight occasions after the morning TMR distribution, and analysed for ASFB contamination. The results showed no increase in the number of spores in the time interval considered: in the first sample, collected at 10:00, the ASFB content in the TMR was 5·05 $\log_{10}$ MPN while in the last sample collected at 18:00 the ASFB content was 5·04 $\log_{10}$ MPN. Similarly, also Vissers et al. (2007b) found no growth of butyric acid bacteria in the feed in the barn.

The hygiene scores of the cows and cubicles are reported as means of the two experimental years in Table 2. The animal hygiene scores differed significantly between the years ($P < 0·05$), there being deterioration in the cleaning condition of udder, leg and flanks in the second year of the experiment. There was an increase in the percentage of leg 3 + 4 scores from 27·1% in the first year to 49·9% in the second (results not shown). In agreement with the findings of Sandrucci et al. (2014), the legs were the dirtiest part of the cows (on average 38·6 ± 3·42% of cows had a hygiene score >3). The cow udders showed, on average, a suboptimal level of cleanliness compared to previous studies (Sandrucci et al. 2014), with a slight, but not significant, decrease in the percentage of udders with score >3 in the last experimental period when the complete milking routine was applied (25·8, 28·1 and 17·7% for the three periods, respectively; Table 2). The percentage of animals with dirty udders is an important aspect in ASFB milk contamination, given the positive correlation between spore contaminated bulk milk and the prevalence of lactating cows with dirty udders on the farm (Nadeau et al. 2010; Zucali et al. 2015). With regard to cubicle cleanliness, no significant difference was noted in the three experimental periods and, as mentioned above, the bedding was renewed weekly, always in the same manner, throughout the two years.

The ASFB faecal content was similar throughout the three periods of the experiment (Table 3) while in the third period the bedding materials showed a limited, but significant ASFB reduction ($P = 0·02$) compared to the first and second periods. However it was the bedding that showed the highest ASFB and SPC values, suggesting that it is an important source of milk contamination. Thus it is essential

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Table 2. Cow hygiene and cubicle cleanliness in the three experimental periods expressed as percentages of scores 3 + 4 for cow hygiene and percentage of score 3 for cubicle cleanliness (least squares means of the two years)

		F	F+Post	Pre+W+F+Post	SE	*P*	1 *vs.* 2	1 *vs.* 3	2 *vs.* 3
Cow flank hygiene	%	20·9	26·1	22·4	2·74	0·35	0·17	0·69	0·34
Cow leg hygiene	%	39·9	41·2	34·5	3·19	0·31	0·76	0·24	0·15
Cow udder hygiene	%	25·8	28·1	17·5	4·39	0·22	0·68	0·19	0·10
Cubicle cleanliness	%	36·1	35·0	28·6	4·79	0·49	0·86	0·34	0·27

F, forestripping; F+Post, forestripping+post-dipping; Pre+W+F+Post, pre-dipping+wiping+forestripping+post-dipping.

Table 3. Anaerobic spore forming bacteria (ASFB) and standard plate count (SPC) contamination in faeces, bedding material, teat swabs and bulk tank milk in the three experimental periods (least squares means of the two years)

	F	F+Post	Pre+W+F+Post	SE	*P*	1 *vs.* 2	1 *vs.* 3	2 *vs.* 3
ASFB								
Faeces (log_{10} MPN/g)	3·53	4·09	3·47	0·30	0·34	0·23	0·91	0·19
Bedding material (log_{10} MPN/g)	4·20	4·19	3·80	0·09	0·03	0·94	0·02	0·02
Teat swabs (log_{10} MPN/swab)	2·20	1·94	1·30	0·12	<0·001	0·13	<0·001	<0·001
Milk (log_{10} MPN/L)	2·47	2·79	1·82	0·17	<0·01	0·19	0·02	<0·01
SPC								
Faeces (log_{10} CFU/g)	6·70	6·79	7·14	0·11	0·06	0·55	0·03	0·06
Bedding material (log_{10} CFUC/g)	9·53	9·54	9·93	0·24	0·46	0·96	0·29	0·30
Teat swabs (log_{10} CFU/swab)	6·88	5·88	5·46	0·23	<0·001	<0·001	<0·001	0·20
Milk (log_{10} CFU/L)	4·51	4·21	3·80	0·16	0·03	0·21	<0·01	0·09
Propionibacteria								
Faeces (log_{10} CFU/g)	3·07	4·69	4·37	0·94	0·51	0·31	0·83	0·40
Bedding material (log_{10} CFU/g)	5·23	5·14	5·8	1·20	0·85	0·95	0·63	0·73
Teat swabs (log_{10} CFU/swab)	2·95	2·59	2·17	0·16	<0·01	0·13	0·07	0·001
Milk (log_{10} CFU/L)	2·22	2·16	1·87	0·24	0·52	0·82	0·27	0·35

F, forestripping; F+Post, forestripping+post-dipping; Pre+W+F+Post, pre-dipping+wiping+forestripping+post-dipping.

that great attention be paid to bedding cleanliness. The number of spores detected on the teat swabs, collected after pre-milking procedures and before cluster attachment, was significantly lower in the last experimental period when pre-dipping, wiping, fore-stripping and post-dipping were adopted ($P < 0·001$), compared to the other two periods. Thus the decreased teat spore count attests to the positive effect of employing pre-dipping, although it must also be recognised that the parallel reduction in the contamination of bedding materials could have contributed to the abatement. The bacterial count on teat swabs decreased significantly with the introduction of post-dipping, but there was no significant additional response to the inclusion of pre-dipping in the milking routine.

The concentration of ASFB in milk, estimated within certain confidence limits through the MPN procedure, ranged between 1·82 and 2·79 log_{10} MPN/L, values similar to those reported by Zucali et al. (2015). Instead, the ASFB content in the milk was significantly reduced on introducing pre-dipping to the milking routine in the third experimental period ($P < 0·01$). Indeed, Magnusson et al. (2006), comparing different teat-cleaning methods, concluded that the most effective method to reduce the milk spore content (96% reduction) was by using a moist washable towel followed by drying with a dry paper towel, for a total time of 20 s per cow. In general, a longer cleaning time and more vigorous methods were more effective in reducing spore contamination. The adoption of pre-dipping and wiping also determined a significant decrease in SPC contamination of milk in the third period, compared to forestripping alone and forestripping plus post-dipping. The introduction of post-dipping in addition to forestripping did not show any significant effect on milk ASFB and SPC. Concerning forestripping, it must be noted that this practice, according to Miller et al. (2015), is associated with an increase in mesophilic spore concentration in bulk tank milk, probably as a consequence of teat contamination during cluster attachment, originated by human hands.

Despite the fact that propionibacteria are widespread in nature, and have been identified in hay, in milk and on the teat surface (Vacheyrou et al. 2011), scant information is available with regard to their presence in faeces and the barn environment. Propionibacteria were present at a high level in the bedding material (>5 log_{10} CFU/g) whereas in raw milk their level was low (Table 3). Even if their content in the milk was slightly reduced by the application of post-dipping, and later by pre-dipping, a statistically significant reduction was observed only on the teat surface when pre-dipping was adopted (Table 3).

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Table 4. Microbiological analyses on raw milk (least squares means of the two years)

	F	F+Post	Pre+W+F+Post	SE	*P*	1 *vs.* 2	1 *vs.* 3	2 *vs.* 3
Coliforms, (log_{10} CFU/mL)	0·95	1·69	1·10	0·21	0·07	0·03	0·63	0·07
Lactic acid bacteria, (log_{10} CFU/mL)	3·54	3·72	3·30	0·19	0·34	0·54	0·39	0·15
Lactic acid bacteria, (% SPC)	78·5	88·1	87·2	3·02	0·08	0·04	0·07	0·82

F, forestripping; F+Post, forestripping+post-dipping; Pre+W+F+Post, pre-dipping+wiping+forestripping+post-dipping.

Table 5. Frequency of different *Clostridium* species detected in faeces, bedding material, teat swabs and milk

	Samples	*C. tyrobutyricum*	*C. beijerinckii*	*C. butyricum*	*C. sporogenes*
Faeces	6	1, 1F+Post	3, 2F+Post; 1Pre+W+F+Post	–	2, 2F
Bedding material	6	1, 1F+Post	2, 2F+Post	4, 1F; 2F+Post; 1Pre+W+F+Post	1, 1F
Teat swabs	30	8, 3F; 5F+Post	3, 3F+Post	5, 1F; 2F+Post; 2Pre+W+F+Post	8, 6F; 1F+Post; 1Pre+W+F+Post
Milk	9	5, 2F; 3F+Post	1, 1F	1, 1 F+Post	1, 1 F

F, forestripping; F+Post, forestripping+post-dipping; Pre+W+F+Post, pre-dipping+wiping+forestripping+post-dipping.

The coliform and lactic acid bacteria (LAB) counts in the milk did not vary significantly in the three milking routines (Table 4). A low LAB content is of important concern in raw milk cheese making, due to the key role LAB play in the acidification of curd and their contribution to cheese aroma and texture (Tormo et al. 2015). According to Mallet et al. (2012) a decrease in LAB was observed in raw milk associated with reduced SPC. The percentage of LAB on total SPC ranged from 78·5 to 88·1% during the three experimental periods, with a slight increase in using post-dipping and pre-dipping procedures, compared with forestripping alone. These values are comparable with findings reported by Brasca et al. (2014) in raw milk (78–94% of SPC). The result suggests that an accurate milking routine does not necessarily reduce milk LAB, which also originate from the udder native bacterial populations within the mammary gland (Al-Qumber & Tagg, 2006).

Clostridium species detected in faeces, bedding materials, teat swabs and milk are shown in Table 5. All the tested species were detected in all the matrices, with exception of *Cl. butyricum* that was not detected in faeces. *C. tyrobutyricum,* the most frequently isolated species on cheeses affected by gas defect, seems to be less frequent in faeces and bedding and more frequent on teat skin and in milk than the other *Clostridium* species. Moreover it is worth noting that *C. tyrobutyricum* was never detected on teats or in milk when the complete milking routine (including pre-dipping, wiping, forestripping and post-dipping) was applied.

Conclusions

The study underlines the key role of teat hygiene in milk spore contamination, which is of utmost importance in the case of raw milk intended for cheese production. An important source of milk contamination lies in the bedding materials, thus suggesting that farmers should pay great attention to its cleanliness. Pre-milking teat dip with a detergent/emollient solution followed by drying with a paper towel significantly reduced the ASFB count on teat skin, and lowered both ASFB and SPC in milk. The introduction of post-dipping in addition to forestripping did not show any significant effect on milk spore content, but it did reduce the milk standard plate count. Interestingly, the implementation of pre-dipping and wiping in the milking routine did not seem to negatively affect the content in the milk of lactic acid bacteria, which play a key role in cheese-making. The high ASFB and SPC levels in the bedding suggest the potential usefulness of more frequent cubicle cleaning and bedding replacement. The study on the *Clostridium* species has suggested that different patterns of species characterise different matrices. Further studies are needed to investigate the dynamics of the *Clostridium* species throughout the entire spore contamination chain.

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