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Management practices and forage quality affecting the contamination of milk with anaerobic spore forming bacteria

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Running title: Management practices, forage quality and milk ~~and cheese~~ anaerobic spore contamination

Management practices and forage quality affecting the contamination of milk ~~and cheese~~ with anaerobic spore forming bacteria

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Abstract

BACKGROUND Anaerobic spore forming bacteria (ASFB) in milk derive from the farm environment and the use of silages and management practices are the main responsible of milk ASFB contamination.

The aim was to evaluate the relationships between feeding, milking routine and cows hygiene and milk and Grana Padano cheese (produced with and without lysozyme) ASFB contamination.

RESULTS The study involved 23 dairy farms. ASFB in corn silage were on average 2.34 ± 0.87 $\log_{10}$ MPN g^{-1}. For grass, Italian ryegrass and alfalfa, ASFB ($\log_{10}$ MPN g^{-1}) were numerically higher for silages (3.22) than hays (2.85). The use of corn silages of high quality (high lactic and acetic acids concentrations) decreased the milk ASFB contamination, whilst the use of herbage silages did not affect it. The presence (>40%) of cows with dirty udders increased the ASFB contamination of milk, while forestripping had a positive effect (-9% ASFB).

Ripened Grana Padano had ASFB count below the analytical limit; *Cl. tyrobutyricum* DNA was found only in wheels produced without lysozyme which also showed late blowing.

CONCLUSION The factors increasing milk spore contamination were corn silage quality, cow udder hygiene and inadequate milking routine. Late blowing was present only in cheeses without lysozyme.

Keyword: silage, *Clostridium*, milk, management practices, spore

INTRODUCTION

It is generally known that the gas-producing anaerobic spore forming bacteria (ASFB) are the main responsible of the defect called late blowing in hard cheeses. Late blowing can lead to off-flavors and excessive gas formation in cheese, due to bacteria ability to convert lactic acid into butyric acid, hydrogen, and carbon dioxide at relatively low pH. Moreover, spores of anaerobic bacteria survive milk pasteurization and pass unaffected into cheese. Among ASFB, *Clostridium tyrobutyricum* is particularly associated with late blowing during the ripening process of hard-cooked-cheeses[1] but also other *Clostridium* species were found responsible for the problem, e.g. *Clostridium sporogenes, Clostridium beijerinckii* and *Clostridium butyricum*.[2,3]

The late blowing is a relevant defect of Grana Padano PDO cheese production: 15% - 35% of total production showed this problem[4] and to inhibit late blowing of cheese the addition of lysozyme to the vat milk (20–30 ppm) is allowed in Grana Padano production.[5] The threshold value of spore concentration in milk causing late blowing is between 600 and 1000 most probable number (MPN) L^{-1} [6] but some authors[4] reported the problem also with a value lower than 100 MPN L^{-1}. The average concentration of spore in milk produced in Lombardy (North Italy) was 220 MPN L^{-1} in 2013.[6]

Spores of butyric acid bacteria in milk derive from the farm environment and the most important source of contamination is the use of silages in the ration[7]; hence, it is

important to determine the spore content of the forages fed to cows but information about concentrations of butyric acid bacterial spores in farm-scale silages is still rather scarce.[8] It is well known that high concentrations of ASFB are associated with anaerobic instability of silage: the creation and maintenance of anaerobic conditions in ensiled forages are important to prevent the growth of aerobic microorganisms but, in practice, exposure of silage to air is unavoidable. The growth of clostridia takes place during the acidification phase at the beginning of silage process[9] and continues during the storage period when small amounts of air can penetrate into the silage, for instance, because silage covers (usually plastic sheets) are not completely airtight.[7] When silage is exposed to air, oxygen penetrates deeply into it, and aciduric aerobic organisms (e.g., yeast) consume oxygen and the substances that inhibit the clostridia growth, i.e., organic acids produced by bacteria anaerobic fermentation. The consumption of both acids and oxygen leads to the development of micro-niches with less inhibitory activity, which might allow the growth of clostridia.[10] In the Po valley corn silage, the main forage used in the total mixed ration (TMR) of dairy cow, has high quality for ensiling because of its relatively high DM content, low buffering capacity, and adequate levels of fermentable sugars.[11] Corn silage cores usually contain less than 3 $\log_{10}$ MPN g^{-1} of butyric acid bacteria spores [9,12] but as demonstrated by Vissers *et al.*[1] spore concentration is significantly higher in corn silage surface layer than in the core. The same authors concluded that on Dutch farms, corn silage was a more important source of ASFB contamination in milk than grass silage.

Although a simulated model proposed by Vissers *et al.*[7] demonstrated that silage contamination is the main source of spore milk content, other identified sources are soil, feces and bedding materials. Spores survive the passage through the digestive tract of the cows and are excreted with the feces. A direct relation between spore content in feces and in milk was obtained by Nadeau *et al.*[13] The same authors also associated the

high milk spore content with poor cleanliness condition of cows. Cow cleanliness entered also in the forecasting model of Vissers *et al.*[7] In addition the preparation of udder and teats plays a role on spore content of milk: Rasmussen *et al.*[14] obtained a significant reduction of spore milk content applying an accurate teat cleaning before cluster attachment, while Vissers *et al.*[7] underlined the efficacy of teat cleaning as a measure to reduce milk contamination. Moreover Arias *et al.*[15] identified the presence of dust on the surfaces of milking parlour and airborne dust as risk factors for the presence of *Clostridium* spores in ewe milk.

The aim of this study is to determine the most important risk factors (feeding and management practices) that affect milk and cheese spore contamination in dairy farms.

MATERIALS AND METHODS

Farm characteristics

A total of 23 farms were chosen from 400 farms belonging to the same milk cooperative in Lombardy, in the North of Italy and their total production was destined to produce Grana Padano PDO cheese. The choice of the 23 farms was based on the following criteria: location in the flatland; lactating cows housing in cubicles; herringbone milking parlour; herds with >50 milking cows; feeding systems based on TMR with or without herbage silages.

Each farm was visited once during the summer 2012 and a questionnaire was filled out to collect information on milking parlour, milking routine, TMR composition, crop characteristics. Information about milk yield and quality of each farm was provided by the milk cooperative. Bulk milk was refrigerated at 11°C after the two daily milkings, following the indication of Grana Padano PDO production rules.

Sampling and analyses

During each visit, the forages (hays and silages) used in the lactating cow TMR and the TMR were sampled. Samples were dried in a forced air oven (55°C for 48h) and ground to pass through a 1 mm sieve using a Wiley mill (Pulverisette 19, Fritsch, Idar-Oberstein, Germany) prior to chemical analyses. Forages and TMR were analyzed for Dry Matter (DM), ash, Crude Protein (CP) and ether extract with the methods of AOAC[16] and starch with the method AOAC[17]; NDF was analyzed with the method of Mertens[18], ADF and ADL with the method of Van Soest *et al.*[19] Extracts from fresh samples of silages were prepared and pH was measured. Silage samples were also analyzed for lactic acid and volatile fatty acids[20] and ammonium-N by direct distillation and titration using a Kjeltec 2300 analyzer (Foss Analytical A/S, Hillerød, Denmark). Samples were analyzed in situ for rumen NDF digestibility at 48 h (NDFD) according to Spanghero *et al.*[21]. Energy value of forages was calculated by NRC[22] equations.

Bulk milk was sampled from the tank at the end of the evening milking the day of the visit, the samples were transported to the laboratory under refrigeration (4°C) no later than 12 h from collection, and immediately subjected to chemical and microbiological analysis.

For microbiological analysis forage samples were chopped for 1 min in a sterile homogenizer, then (10 g) suspended in a 1:10 peptone salt solution (PPS; 1 g of bacteriological peptone and 9 g of sodium chloride per liter), and homogenized twice for 2 min at maximum speed using a Stomacher (BagMixer 400, Interscience). The raw milk samples were analysed directly.

Gas-forming anaerobic spores and propionibacteria were determined in forage samples. Anaerobic spore content was obtained throughout the MPN. MPN enumeration was performed with three 10-fold dilutions with three tubes at each dilution. The culture medium used for MPN was prepared with reconstituted skimmed milk (10% wt/v) supplemented with a solution of yeast extract (1.0%), sodium lactate (3.36%), sodium

acetate (1.0%), cysteine (0.2%) with vaseline/paraffin (1:1, wt/wt) seals. The heat treatment applied to the inoculated milk medium was 80°C for 10 min. The incubation period was 7 days at 37°C. In order to detect the presence of *Clostridium* species (*Cl. tyrobutyricum, Cl. butyricum, Cl. beijerinckii; Cl. sporogenes*,) positive tubes were analyzed by multiplex PCR according to Cremonesi *et al*.[23] P2 agar containing peptone 5 g; beef extract, 3 g; yeast extract, 5 g; sodium lactate, 1 g; agar 15 g per litre[24] was used for anaerobic enumeration of propionibacteria at 30°C for 7 days. For incubation in anaerobic conditions, jars with anaerocult A (Merk KGaA, Darmstadt, Germany) were used.

Milk bulk samples were analyzed for fat, protein, lactose, urea using a Fourier transform infrared analyser (Milkoscan FT6000; Foss Analytical A/S). Somatic cell counts were determined by Fossomatic SC.

Milk bulk samples were also examined for standard plate count (SPC) and coliform count (CC) using Petrifilm (3M Canada, London, Ontario, Canada) and plates were incubated respectively at 30°C for 72 and 24 h. Lactic acid bacteria (LAB) were determined on de Man Rogosa and Sharpe (MRS) agar (Biolife, Milan, Italy); the plates were incubated anaerobically at 30°C for 72 h. Propionibacteria and anaerobic spore content were determined as previously described.

Cow cleanliness

Hygiene scores of dairy cows were assessed through direct observation in the milking parlour at each farm visit according to Schreiner and Ruegg[25]: udder, flanks and legs of each milked cow (4216 cows) were scored in the same way based on a 4-point scale system, where score 1 indicates very clean skin while score 4 indicates skin completely covered with dirt. Later on the percentage of animals with score 3 and 4 for udder, flanks and legs was calculated for each farm.

Cheese production

The farms were divided into two groups (HIGH and LOW) on the basis of the spore forming count in milk and the threshold value used to divide the bulk milk was 2.5 $\log_{10}$ MPN L^{-1}. The milk of the two groups was separately collected and within each group it was used for two different Grana Padano cheese production with and without the addition of lysozyme.

Grana Padano cheese was made with milk partially skimmed by natural creaming process. The milk was transferred to copper bell-shaped vats containing 1000 L and a natural whey culture was added as a starter. Milk was then heated to 32 °C, calf rennet was added. The resulting curd was broken up into small granules to the size of grains of rise after 10 min from the rennet addition; it was then cooked by increasing the temperature to 54-55 °C in about 10 min and constantly stirred. The broken curd was then left to rest maintaining the temperature at 53 °C for 60 min. The cheese mass was then cut in two wheels and transferred into traditional moulds for 2 days, salted in brine for 22 days and ripened at 15-22 °C .

Sixteen cheese wheels were obtained: 8 with milk of HIGH group (4 with lysozyme and 4 without addition) and 8 with milk of LOW group (4 with lysozyme and 4 without addition). The presence of *Clostridium* species and anaerobic spore content in cheese samples were determined as previously described. X-Ray analysis was performed for cheese wheels produced with and without the addition of lysozyme from HIGH and LOW milk group. X-ray analysis, commonly used in dairy plants to detect defects due to bacterial fermentation in Grana Padano cheese, was performed at 7 months of ripening following the indications of the Quality control handbook of the cheese plant.

Statistical analysis

Bacterial counts were expressed as base 10 logarithm, for statistical analysis.

Relationships among farm dimension, use of forestripping procedure, use of predipping procedure, cow udder hygiene, microbiological quality of raw milk (standard plate count, lactic acid bacteria, anaerobic spores, propionibacteria and coliform counts), anaerobic spores in corn silage and in TMR, silage propionic acid, silage acetic acid, silage lactic acid were evaluated through a multiple correspondence analysis (Proc CORRESP; SAS[26],) to find a two dimensional graphical representation of the rows and columns of a contingency table.

Difference in chemical composition and nutritive value between silage (with the exclusion of corn silage) and hay samples and among forage species were tested by Proc GLM[26] as follows:

$Yijk = \mu + Ci + Fj + Ci*Fj + eijk$

Where:

Yijk = dependent variables;

μ = general mean;

Ci = conservation effect (i=1-2; silage vs hay);

Fj = forage species effect (J=1-3; alfalfa, grass and Italian ryegrass);

eijk= residual error.

The characteristics of the farms based on LOW or HIGH milk spore forming count were identified by Proc GLM[26] with the following model:

$Yijkl = \mu + Gi + Dj + Sk + eijkl$

Where:

Yijkl = dependent variables;

μ = general mean;

G_i= spore count in bulk milk effect (i=1-2; <2.5 $\log_{10}$ MPN L^{-1} or ≥ 2.5 $\log_{10}$ MPN L^{-1});

D_J= farm dimension in term of dairy cows number (j=1-2; <100 or ≥ 100);

S_k= corn silage, % on total DM (k=1-2; <37% or ≥37%);

eijkl = residual error.

Data are reported as Least Square Means

RESULTS AND DISCUSSION

Chemical composition, nutritive value and anaerobic spore forming count of forages

The average forage content in the lactating cow TMR was 560 ± 49 g kg^{-1} DM. The amount of forage in TMR was adequate and there was a low variability among farms for this parameter (CV=8.8%). Forage quality is a key factor to maintain high quality milk production.[2,3,8] However, increasing forage proportion, especially silages, could be related to a higher risk of milk spore contamination.[1,27] In the present study, corn silage was included in the TMR of all farms (on average 363 g kg^{-1} DM) whilst other silages (grass, Italian ryegrass or alfalfa) were used in half of the farms surveyed (48%). Particularly, grass and Italian ryegrass silages were used in 30% of farms and alfalfa silage was used in 26% of farms. The amount of silages, other than corn silage, was on average 105 ± 57 g kg^{-1} DM with a wide range of variation (42-262 g kg^{-1}).

Average chemical composition, nutritive value and NDFD of corn silages were: ash 50.3, CP 77.9, EE 32.8, NDF 454, ADF 294, ADL 45.9, starch 298 and NFC 385 g kg^{-1} DM, NE_l 6.54 MJ kg^{-1} DM (expressed at 3X level of maintenance) and NDFD 498 g kg^{-1} NDF. The DM content was on average 351 g kg^{-1}. Average ASFB (log_{10} MPN /g^{-1}) was 2.34 ±0.87 with a great variability among samples (CV=38%). The average ASFB was slightly lower than the value (2.75) reported by Borreani and Tabacco[28] for corn silages sampled in the peripheral areas of the silos in commercial Italian farms and equal to the value (2.34) reported by Colombari *et al.*[9]. As demonstrated by Danner *et al.*[29] under constant conditions, the aerobic stability of silages is determined by the

concentration of acetic acid; in the present study the acetic acid content in corn silage was 19.9 ± 8.60 g kg^{-1} DM with a wide variability (CV=43.1%) and this could have affected the aerobic stability of silages and consequently the spore content. Average corn silage pH was 3.96 ± 0.18 and the lactic acid content was 48.2 ± 13.6 g kg^{-1} DM whilst no significant levels of butyric acid were detected.

Chemical composition, NDFD and nutritive value of grass, Italian ryegrass, alfalfa hays and silages are reported in table 1. Silages had higher ash and CP contents (g kg^{-1} DM) than hays (ash: 71.3 vs 112, P<0.001 and CP: 101 vs 129, P=0.05, for hay and silage respectively) whilst hays had higher NDF content (557 vs 496 g kg^{-1} DM, P=0.007, respectively for hays and silages). Silages were also characterized by higher NDFD values than hays (394 vs 476 g kg^{-1} DM, P=0.002 for hay and silage, respectively). These differences result in a higher organic matter digestibility (OMd) and net energy for lactation (NE_l) for silages than hays (OMd = 702 vs 642 g kg^{-1} DM, P=0.01, for silage and hay, respectively; NE_l= 4.86 vs 4.26 MJ kg^{-1} DM, P=0.02; for silage and hay, respectively). Overall, the mean values were low showing a poor quality of the forages used in the TMR.

As previously reported, silages were ensiled as wrapped bale silages and the forage was not chopped; wrapping silage offers many advantages compared to hay making: large quantities of forage can be conserved in a short time, forage conservation is less weather-dependent, and silage can be easily mechanized. On the other hand, the disadvantages include an increased risk of moldy and mycotoxin production, an increased risk of listeriosis, clostridial spoilage, and the loss of nutrients due to difficulties in achieving the basic conditions required for stability during the storage of silage.[30] Furthermore, it has to be underlined that usually baled silages underwent a more restricted fermentation than chopped silages ensiled in bunker silos and that less anaerobic conditions are usually obtained with baled silages than chopped silages. In the

present study, ASFB tended to be higher for silages (alfalfa, Italian ryegrass and grass) than hays (3.22 vs 2.85 $\log_{10}$ MPN g^{-1} for silage and hay samples, respectively, P=0.09) but differences were not detected among forages. Grass species silages however had a higher ASFB spore content than corn silage (3.22 vs 2.75 $\log_{10}$ MPN g^{-1} respectively). Concerning alfalfa silage, the average ASFB (3.00 $\log_{10}$ MPN g^{-1}) was higher than the value (2.11) reported by Colombari *et al.*[9] for a sample with a similar DM. On average, alfalfa silages had a very high pH value (6.03) and a low lactic acid concentration (9.8 g kg^{-1} DM). Similarly, contents of ASFB in silages were not in the range of those reported for grass chopped herbages and for well-conserved haylage.[2,31,32] Furthermore, differently from what reported by Julien *et al.*[2] clostridia were detected in hay samples. Information about concentrations of butyric acid bacteria spores in farm-scale silages is rather scarce. Studies in France conducted in the 1970s showed that about 20% of grass silages contained more than 10^5 butyric acid bacteria spores, indicating a poor quality of forages[8] and in agreement with the results of the present study.

Overall, average ASFB content for TMR was 4.75 ±0.73 $\log_{10}$ MPN g^{-1} and higher than the values estimated from the ASFB spore content of the forages.

Effect of silage quality and management practices on anaerobic spore contamination of milk

The ASFB content in bulk tank milk of farms that used other ensiled forages in the diet, in addition to corn silage (2.73 $\log_{10}$ MPN L^{-1}), did not differ much from the content of farms that used only corn silage (2.78 $\log_{10}$ MPN L^{-1}) (Table 2). This result was probably due to the small quantity of other silages used in the diet (105 g kg^{-1} DM on average); the results of the present experiment are consistent with a study of Vissers *et al.*[1] which demonstrated that on Dutch farms, corn silage was a more important source of ASFB contamination in milk than grass silage.

Table 2 shows farm distribution and ASFB values in bulk tank milk considering corn silages quality. Farms that used high quality corn silage (lactic acid >50 g kg^{-1} DM, acetic acid >20 g kg^{-1} DM, propionic acid <1.5 g kg^{-1} DM) had a lower spore count in bulk tank milk than the others farms. The concentration of propionic acid in the corn silage seems to influence more the bacterial contamination of milk: farms with low propionic acid content in the corn silage had 13.8% less ASFB in milk than the other farms. All corn silage pH values were below the threshold of 4.2, which can indicate fermentative problems of the silage. High silage pH could be due to: high dry matter at ensiling (> 50% DM), cold weather during harvest, slow or poor packing, silage with excess ammonia or urea, clostridial contamination, spoiled or moldy silages, silages containing manure.[33]

Farms characterized by lower ASFB content in corn silage and TMR produced milk with lower level of anaerobic spores than the other farms (table 2). A linear regression analysis indicated that there was a positive relationship between milk ASFB (log_{10} MPN L^{-1}) and corn silage ASFB (log_{10} MPN g^{-1}) content (y= 0.294*ASFB corn silage + 2.075; r^2=0.217). It is well known that high concentrations of anaerobic spores are associated with anaerobic instability of silage during the primary fermentation phase.[8] However, as recently demonstrated by Vissers *et al.*[1] milk spore contamination is mainly related to aerobic instability rather than to anaerobic instability problems; therefore, also corn silage, being subjected to aerobic deterioration, has an important effect on milk spore contamination.

Management practices, in particular during milking, can influence the quality of bulk tank milk as found by Rasmussen *et al.*[14]. In the present study (table 3) farms that paid more attention to milking routine (use of gloves, forestripping, predipping) achieved the best results in terms of a lower ASFB milk contamination. In particular, farms that carried out forestripping had -9% of ASFB in milk. Furthermore, farms that used the

complete milking routine operations (use of gloves, dry udder clean, forestripping, predipping, postdipping), achieved the lowest ASFB milk contamination. These results are consistent with those of Zucali *et al.*[34] who found that the dairy farms which used an accurate milking routine produced milk with the best microbiological quality in terms of standard plate count, propionibacteria and coliform count.

Cow hygiene, in particular of the udder, influenced milk ASFB content as found by some authors.[7,13] The presence of more than 40% of animals with dirty udders in farms increased the average ASFB contamination of milk by 15%.

Table 4 shows the frequency of different *Clostridium* species in the forages and in the TMR collected in the study. The frequency of *C. tyrobutyricum* was the highest, in particular in corn silage (66.7%), grass hay (77.8%) and TMR samples (82.6%). *C. beijerinckii* was the second species for presence in feeds, particularly in TMR samples (47.5%). The almost constant detection of *C. tyrobutyricum* in the sampled forages arises some concern because, as demonstrated by Klijn *et al.*[35], late blowing in Gouda cheese was exclusively associated with the growth of *C. tyrobutyricum* and not with the presence of *C. beijerinckii* or *C. sporogenes*. In five samples were ASFB were present, none of the tested clostridia was detected. The presence of other Clostridia can be hypothesized; for example Rossi and Dellaglio[36] detected *C. saccharolyticum* and *C. baratii* in silages whilst Julien *et al.*[2] identified *C. disporicum* as another predominant member of clostridial population in silage. Clostridial species present in forages were consistent with milk spore contamination ~~confirming the strict relationship~~.

All the tested species were detected in milk samples.

Figure 1 shows the results of multiple correlations between variables. The correspondence analysis divided the variables in four groups. The groups identified different farm situations based on management practices and milk and feeds quality, defined as follows: 1) good management (upper left); 2) low quality of feeds and milk

(upper right); 3) high quality of feeds and milk (lower left); 4) bad management (lower right). Farms characterized by good management (group 1) had adequate milking routine (forestripping and predipping procedures), sufficient cleanliness of the animals (less than 40% of the herd had dirty udder), high lactic acid content in corn silage (>50 g kg^{-1} DM), and low coliform content (<2.2 log_{10} CFU mL^{-1}). These farms had the highest number of cows (≥100 dairy cows), and probably adopted a better labor organization. Farms with high quality milk (low spore count) (group 2) had also low value of SPC and LAB. Lactic acid bacteria content of all farms milk is correlated with SPC (r=0.5293; P <0.01), which is correlated with bacterial spore content in milk (r=0.534; P<0.01). Corn silage quality of these farms was good, in terms of propionic and acetic acid contents (<1.5 g kg^{-1} DM, and >20 g kg^{-1} DM, respectively) and anaerobic spore presence in corn silage and TMR (<2.3 log_{10} MPN g^{-1}, <4.7 log_{10} MPN g^{-1}).

Milk and cheese quality

The ASFB content in milk was 3.14 and 2.33 log_{10} MPN L^{-1} respectively for the HIGH and the LOW groups. The characteristics of the farms belonging to the two groups are showed in table 5. The average milk yield of the cows of farms of the HIGH group was lower than that of the LOW group (P<0.01). No differences between the two groups were observed in the composition and quality of the TMR and in the quality of corn silage. Despite this, the average spore content of corn silage differed between HIGH and LOW groups (2.47 *vs* 1.90 log_{10} MPN g^{-1}, P < 0.07); this could explain the different level of milk contamination. This finding is again consistent with the results of Visser *et al.*[1] who showed a close linkage between corn silage and milk contamination. Milking cows were dirtier in HIGH group than in LOW group; in particular legs and flanks showed the worst results. ~~with a significant difference between groups.~~ SPC and ~~lactic~~

~~acid bacteria~~LAB counts did not differ significantly between the groups, ~~while~~ propionibacteria and CC were ~~significantly~~ higher in LOW group but not significantly. The milk of LOW and HIGH farm groups was separately collected and cheesemaking trials were carried out in a cheese factory that usually produces Grana Padano cheese. The milk of each group was further divided into two halfs: in one half lysozyme was added into the vat (25 mg L^{-1}) while no addition was done in the other one. After 7 months of ripening, even if spore count was below the analytical limit (<30 MPN g^{-1}), ~~in all the experimental cheeses both in HIGH and LOW group,~~ the prevalence of *Cl. tyrobutyricum* was highlighted, by DNA assay, only in cheeses produced without lysozyme, both in LOW and HIGH groups. *Cl. tyrobutyricum* outgrowth and butyric acid fermentation were confirmed by X-Ray analysis evidencing late blowing defects. It is worthwhile noting that widespread blowing was ~~significantly~~ lower in cheeses produced with the LOW group milk (Figure 2).

CONCLUSIONS

The study underlines the relation among forage quality, dairy farm management practices and milk and cheese ASFB contamination. The main risk factors which increase milk spore contamination were spore content of corn silage, scarce cow udder hygiene and inadequate milking routine (lack of forestripping and predipping procedures). The presence in the TMR of herbage silages (107 g kg^{-1} DM on average), did not affect significantly milk spore contamination. In Grana Padano cheese *Cl. tyrobutyricum* was highlighted after 7 months of ripening only in the wheels produced without addition of lysozyme~~but the spore caused late blowing only in the wheels produced without addition of lysozyme~~, confirming the effectiveness of this additive in preventing spore outgrowth by clostridia.

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Table 1. Chemical composition, nutritive and anaerobic bacterial spore count of forages (Least square means values)

	n	DM g kg^{-1}	Ash g kg^{-1} DM	EE g kg^{-1} DM	CP g kg^{-1} DM	NDF g kg^{-1} DM	ADF g kg^{-1} DM	ADL g kg^{-1} DM	ASFB log_{10}MPN g^{-1}	NE_l 3x MJ kg^{-1} DM	NDFD g kg^{-1}
Alfalfa											
silage	6	564b	97.3b	19.5bc	184a	456	397b	99.7b	3.00	4.88a	383b
hay	3	924a	70.3c	13.1cd	138b	559	498a	129a	2.30	3.63b	281c
Grass											
silage	2	394c	133a	24.0ab	90.0cd	471	360b	56.5d	3.46	4.75a	466ab
hay	18	916a	76.2c	15.6cd	89.7d	557	360b	57.6d	2.59	4.90a	488ab
Italian ryegrass											
silage	6	412c	105b	30.2a	113bc	577	365b	73.8cd	3.28	4.95a	578a
hay	2	927a	67.3c	11.6d	82.9d	554	396b	86.9bc	2.81	4.25ab	391b
SE[e]		47.3	9.60	3.40	15.7	36.1	34.8	12.8	0.66	0.39	47.9
P[f] forage species		0.08	0.01	0.16	<0.001	0.100	0.004	<0.001	0.65	0.15	<0.001
P type of conservation		<0.001	<0.001	<0.001	0.05	0.007	0.04	0.07	0.09	0.02	0.007
P forage*conservation		0.10	0.13	0.04	0.13	0.10	0.14	0.34	0.91	0.06	0.02

DM, dry matter; EE, ether extract; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; ASFB, anaerobic spore forming bacteria; NE_l 3x, net energy of lactation computed three times at maintenance; NDFD, rumen neutral detergent fiber digestibility with an incubation time of 48 hours.

[a,b,c,d] Least square means within column with different superscript correspond to significant difference for $P < 0.05$

[e]SE: standard error

[f]P: probability

Table 2. Farm distribution and anaerobic spore forming bacteria count in bulk tank milk (log_{10} MPN L^{-1}) considering silage quality

		Anaerobic spore forming bacteria count (log_{10} MPN[a] L^{-1} milk)	
	No. farms	Mean	SD[b]
Farms size			
lactating cows < 100	12	2.78	0.60
lactating cows > 100	11	2.72	0.45
Grass and alfalfa silage			
NO	11	2.78	0.37
YES	12	2.73	0.65
Corn silage quality			
pH < 3.8	4	3.02	0.36
pH > 3.8	19	2.70	0.54
lactic acid < 50 g kg^{-1} DM[c]	10	2.88	0.31
lactic acid > 50 g kg^{-1} DM	13	2.65	0.64
acetic acid < 20 g kg^{-1} DM	12	2.85	0.38
acetic acid > 20 g kg^{-1} DM	11	2.65	0.66
propionic acid < 1.5 g kg^{-1} DM	13	2.60	0.52
propionic acid > 1.5 g kg^{-1} DM	10	2.96	0.48
N_NH_3 corn silage < 10% total N	10	2.78	0.40
N_NH_3 corn silage > 10% total N	13	2.73	0.62
TMR[d] spore count			
log_{10} MPN g^{-1} < 4.7	10	2.61	0.57
log_{10} MPN g^{-1} > 4.7	13	2.86	0.48
Corn silage spore count			
log_{10} MPN g^{-1} < 2.3	10	2.49	0.53
log_{10} MPN g^{-1} > 2.3	11	3.00	0.40

[a]MPN: Most probable number
[b]SD: standard deviation
[c] DM: dry matter
[d] TMR: total mixed ration

Table 3. Farm distribution and anaerobic spore forming bacteria count in bulk tank milk (log_{10} MPN L^{-1}) considering management practices

		Anaerobic spore forming bacteria count log_{10} MPN[a] L^{-1} milk	
	No. farms	Mean	SD[b]
Udder hygiene			
dirty udder < 40% [c]	13	2.58	0.49
dirty udder > 40%	10	2.98	0.51
Milking routine			
use of gloves: NO	12	2.81	0.51
use of gloves: YES	11	2.7	0.56
Dry udder clean			
NO	7	2.73	0.36
YES	16	2.76	0.6
Forestripping			
NO	9	2.92	0.51
YES	14	2.65	0.53
Predipping			
NO	14	2.79	0.49
YES	9	2.69	0.6
Postdipping			
NO	8	2.76	0.58
YES	15	2.75	0.52
Milking routine: no. operations			
0	1	2.69	.
1	6	2.81	0.67
2	2	2.78	0.37
3	3	3	0.44
4	9	2.74	0.31
5	2	2.27	1.36

[a]MPN: most probable number
[b]SD: standard deviation
[c]Dirty udder: % of udder with hygiene score 3 and 4

Table 4. Frequency of different *Clostridium* species detected in forage, TMR and milk samples.

		Cl. beijerinckii	*Cl. butyricum*	*Cl. sporogenes*	*Cl. tyrobutyricum*
	n	yes	yes	yes	yes
Corn silage	21	5	5	2	14
Grass hay	18	4	5	6	14
Grass silage	2	-	-	1	2
Alfalfa hay	3	-	-	1	1
Alfalfa silage	6	2	1	1	4
Italian ryegrass hay	2	-	-	1	1
Italian ryegrass silage	6	-	-	1	3
TMR	23	10	7	1	19
Milk	23	9	6	2	15

Table 5. Characteristics of the farms classified on the basis of HIGH or LOW milk anaerobic spore forming bacteria (Least square means)

	HIGH	LOW	SE[a]	P[b]
Farms, no.	12	11		
Farm size, no. lactating cows	98.5	109	7.31	0.33
Total farm land, ha	49.5	42.3	6.67	0.45
Milk production				
Milk yield, cow day^{-1}	23.3	28.2	1.25	0.01
Fat, g kg^{-1}	38.9	38.8	0.43	0.83
Protein, g kg^{-1}	35.1	35.0	0.21	0.75
Lactose, g kg^{-1}	50.0	50.1	0.17	0.48
Urea, mg dL^{-1}	20.6	21.6	1.08	0.50
Linear Score	4.23	4.31	0.12	0.63
TMR composition				
Forage, g kg^{-1} DMI[c]	565	554	16.0	0.64
Grass silage, g kg^{-1} DMI	59.6	51.5	21.4	0.79
Corn silage, g kg^{-1} DMI	362	364	11.2	0.88
Forages spore contamination				
Corn silage, $\log_{10}$ MPN[d] g^{-1}	2.47	1.90	0.21	0.07
Grass, $\log_{10}$ MPN g^{-1}	2.71	2.11	0.36	0.22
TMR, $\log_{10}$ MPN g^{-1}	4.78	4.51	0.21	0.39
Hygiene Score				
Udder, % 3+4	49.6	31.3	7.78	0.11
Legs, % 3+4	70.0	49.1	8.84	0.11
Flanks, % 3+4	55.5	36.6	8.33	0.12
Milking routine, no. operations	3.00	2.63	0.50	0.61
Milk bacterial counts				
Spore count, $\log_{10}$ MPN L^{-1}	3.14	2.33	0.11	0.00
SPC[e], $\log_{10}$ CFU[f] mL^{-1}	4.21	4.09	0.27	0.75
Coliform, $\log_{10}$ CFU mL^{-1}	1.76	2.54	0.29	0.07
Lactic acid bacteria, $\log_{10}$ CFU mL^{-1}	3.83	3.77	0.14	0.74
Propionibacteria, $\log_{10}$ CFU mL^{-1}	1.85	2.16	0.23	0.36

[a]SE: standard error
[b]P: probability
[c]DMI: dry matter intake
[d]MPN: most probable number

[e]SPC: standard plate count
[f]CFU: colony forming unit

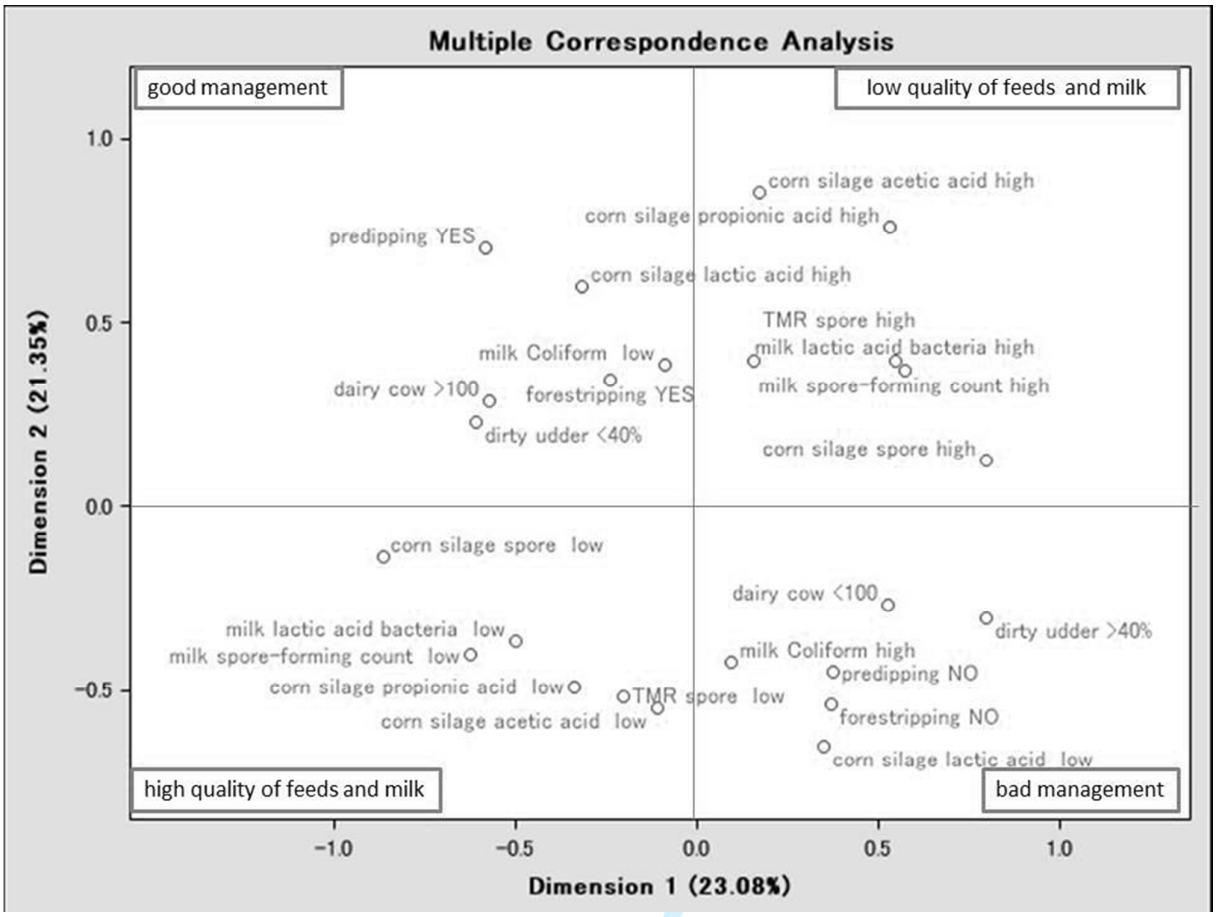

Figure 1. Multiple corrispondence analysis of risk factors for spore contamination in milk of 23 farms

Figure 2. X-Ray analysis of cheese wheels produced WITH and WITHOUT the addition of lysozyme from HIGH and LOW milk group