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bacteria**

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Key Words:	silage , Clostridium , milk, management practices, spore

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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 $2.34 \pm 0.87 \log_{10}$ 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.

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6 **CONCLUSION** The factors increasing milk spore contamination were corn silage
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8 quality, cow udder hygiene and inadequate milking routine. Late blowing was present
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10 only in cheeses without lysozyme.
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14 **Keyword:** silage, *Clostridium*, milk, management practices, spore
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17 18 **INTRODUCTION**

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20 It is generally known that the gas-producing anaerobic spore forming bacteria (ASFB)
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22 are the main responsible of the defect called late blowing in hard cheeses. Late blowing
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24 can lead to off-flavors and excessive gas formation in cheese, due to bacteria ability to
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26 convert lactic acid into butyric acid, hydrogen, and carbon dioxide at relatively low pH.
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28 Moreover, spores of anaerobic bacteria survive milk pasteurization and pass unaffected
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30 into cheese. Among ASFB, *Clostridium tyrobutyricum* is particularly associated with
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32 late blowing during the ripening process of hard-cooked-cheeses¹ but also other
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34 *Clostridium* species were found responsible for the problem, e.g. *Clostridium*
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36 *sporogenes*, *Clostridium beijerinckii* and *Clostridium butyricum*.^{2,3}
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38 The late blowing is a relevant defect of Grana Padano PDO cheese production:
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40 15% - 35% of total production showed this problem⁴ and to inhibit late blowing of
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42 cheese the addition of lysozyme to the vat milk (20–30 ppm) is allowed in Grana
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44 Padano production.⁵ The threshold value of spore concentration in milk causing late
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46 blowing is between 600 and 1000 most probable number (MPN) L⁻¹⁶ but some authors⁴
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48 reported the problem also with a value lower than 100 MPN L⁻¹. The average
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50 concentration of spore in milk produced in Lombardy (North Italy) was 220 MPN L⁻¹ in
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52 2013.⁶

53 Spores of butyric acid bacteria in milk derive from the farm environment and the most
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55 important source of contamination is the use of silages in the ration⁷; hence, it is
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6 important to determine the spore content of the forages fed to cows but information
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8 about concentrations of butyric acid bacterial spores in farm-scale silages is still rather
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10 scarce.⁸ It is well known that high concentrations of ASFB are associated with
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12 anaerobic instability of silage: the creation and maintenance of anaerobic conditions in
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14 ensiled forages are important to prevent the growth of aerobic microorganisms but, in
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16 practice, exposure of silage to air is unavoidable. The growth of clostridia takes place
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18 during the acidification phase at the beginning of silage process⁹ and continues during
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20 the storage period when small amounts of air can penetrate into the silage, for instance,
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22 because silage covers (usually plastic sheets) are not completely airtight.⁷ When silage
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24 is exposed to air, oxygen penetrates deeply into it, and aciduric aerobic organisms (e.g.,
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26 yeast) consume oxygen and the substances that inhibit the clostridia growth, i.e.,
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28 organic acids produced by bacteria anaerobic fermentation. The consumption of both
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30 acids and oxygen leads to the development of micro-niches with less inhibitory activity,
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32 which might allow the growth of clostridia.¹⁰ In the Po valley corn silage, the main
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34 forage used in the total mixed ration (TMR) of dairy cow, has high quality for ensiling
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36 because of its relatively high DM content, low buffering capacity, and adequate levels
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38 of fermentable sugars.¹¹ Corn silage cores usually contain less than $3 \log_{10}$ MPN g^{-1} of
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40 butyric acid bacteria spores^{9,12} but as demonstrated by Vissers *et al.*¹ spore
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42 concentration is significantly higher in corn silage surface layer than in the core. The
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44 same authors concluded that on Dutch farms, corn silage was a more important source
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46 of ASFB contamination in milk than grass silage.

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

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

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16 The aim of this study is to determine the most important risk factors (feeding and
17 management practices) that affect milk and cheese spore contamination in dairy farms.
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21 22 23 24 25 26 27 **MATERIALS AND METHODS**

28 29 **Farm characteristics**

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31 A total of 23 farms were chosen from 400 farms belonging to the same milk cooperative
32 in Lombardy, in the North of Italy and their total production was destined to produce
33 Grana Padano PDO cheese. The choice of the 23 farms was based on the following
34 criteria: location in the flatland; lactating cows housing in cubicles; herringbone milking
35 parlour; herds with >50 milking cows; feeding systems based on TMR with or without
36 herbage silages.
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39 Each farm was visited once during the summer 2012 and a questionnaire was filled out
40 to collect information on milking parlour, milking routine, TMR composition, crop
41 characteristics. Information about milk yield and quality of each farm was provided by
42 the milk cooperative. Bulk milk was refrigerated at 11°C after the two daily milkings,
43 following the indication of Grana Padano PDO production rules.
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53 54 55 **Sampling and analyses**

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6 During each visit, the forages (hays and silages) used in the lactating cow TMR and the
7 TMR were sampled. Samples were dried in a forced air oven (55°C for 48h) and ground
8 to pass through a 1 mm sieve using a Wiley mill (Pulverisette 19, Fritsch, Idar-
9 Oberstein, Germany) prior to chemical analyses. Forages and TMR were analyzed for
10 Dry Matter (DM), ash, Crude Protein (CP) and ether extract with the methods of
11 AOAC¹⁶ and starch with the method AOAC¹⁷; NDF was analyzed with the method of
12 Mertens¹⁸, ADF and ADL with the method of Van Soest *et al.*¹⁹ Extracts from fresh
13 samples of silages were prepared and pH was measured. Silage samples were also
14 analyzed for lactic acid and volatile fatty acids²⁰ and ammonium-N by direct distillation
15 and titration using a Kjeltec 2300 analyzer (Foss Analytical A/S, Hillerød, Denmark).

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26 Samples were analyzed in situ for rumen NDF digestibility at 48 h (NDFD) according
27 to Spanghero *et al.*²¹. Energy value of forages was calculated by NRC²² equations.

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

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60 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

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6 acetate (1.0%), cysteine (0.2%) with vaseline/paraffin (1:1, wt/wt) seals. The heat
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8 treatment applied to the inoculated milk medium was 80°C for 10 min. The incubation
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10 period was 7 days at 37°C. In order to detect the presence of *Clostridium* species (*Cl.*
11
12 *tyrobutyricum*, *Cl. butyricum*, *Cl. beijerinckii*; *Cl. sporogenes*), positive tubes were
13
14 analyzed by multiplex PCR according to Cremonesi *et al.*²³ P2 agar containing peptone
15
16 5 g; beef extract, 3 g; yeast extract, 5 g; sodium lactate, 1 g; agar 15 g per litre²⁴ was
17
18 used for anaerobic enumeration of propionibacteria at 30°C for 7 days. For incubation
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20 in anaerobic conditions, jars with anaerocult A (Merk KGaA, Darmstadt, Germany)
21
22 were used.

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24 Milk bulk samples were analyzed for fat, protein, lactose, urea using a Fourier transform
25
26 infrared analyser (Milkoscan FT6000; Foss Analytical A/S). Somatic cell counts were
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28 determined by Fossomatic SC.

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30 Milk bulk samples were also examined for standard plate count (SPC) and coliform
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32 count (CC) using Petrifilm (3M Canada, London, Ontario, Canada) and plates were
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34 incubated respectively at 30°C for 72 and 24 h. Lactic acid bacteria (LAB) were
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36 determined on de Man Rogosa and Sharpe (MRS) agar (Biolife, Milan, Italy); the plates
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38 were incubated anaerobically at 30°C for 72 h. Propionibacteria and anaerobic spore
39
40 content were determined as previously described.

41 **Cow cleanliness**

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43 Hygiene scores of dairy cows were assessed through direct observation in the milking
44
45 parlour at each farm visit according to Schreiner and Ruegg²⁵: udder, flanks and legs of
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47 each milked cow (4216 cows) were scored in the same way based on a 4-point scale
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49 system, where score 1 indicates very clean skin while score 4 indicates skin completely
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51 covered with dirt. Later on the percentage of animals with score 3 and 4 for udder,
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53 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 rice 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.

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6 Relationships among farm dimension, use of forestripping procedure, use of predipping
7 procedure, cow udder hygiene, microbiological quality of raw milk (standard plate
8 count, lactic acid bacteria, anaerobic spores, propionibacteria and coliform counts),
9 anaerobic spores in corn silage and in TMR, silage propionic acid, silage acetic acid,
10 silage lactic acid were evaluated through a multiple correspondence analysis (Proc
11 CORRESP; SAS²⁶), to find a two dimensional graphical representation of the rows and
12 columns of a contingency table.
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16 Difference in chemical composition and nutritive value between silage (with the
17 exclusion of corn silage) and hay samples and among forage species were tested by Proc
18 GLM²⁶ as follows:
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$$20 \quad Y_{ijk} = \mu + C_i + F_j + C_i * F_j + e_{ijk}$$

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22 Where:

23 Y_{ijk} = dependent variables;

24 μ = general mean;

25 C_i = conservation effect ($i=1-2$; silage vs hay);

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

27 e_{ijk} = residual error.
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41 The characteristics of the farms based on LOW or HIGH milk spore forming count were
42 identified by Proc GLM²⁶ with the following model:
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$$44 \quad Y_{ijkl} = \mu + G_i + D_j + S_k + e_{ijkl}$$

45
46 Where:

47 Y_{ijkl} = dependent variables;

48 μ = general mean;

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

50 D_j = farm dimension in term of dairy cows number ($j=1-2$; <100 or ≥ 100);
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S_k = corn silage, % on total DM ($k=1-2$; $<37\%$ or $\geq 37\%$);

e_{ijkl} = 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 $560 \pm 49 \text{ g kg}^{-1}$ 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 $105 \pm 57 \text{ g kg}^{-1}$ DM with a wide range of variation ($42-262 \text{ g kg}^{-1}$).

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_1 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 ($\log_{10} \text{MPN}_{4g^{-1}}$) 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 Tabacco²⁸ 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

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6 concentration of acetic acid; in the present study the acetic acid content in corn silage
7 was $19.9 \pm 8.60 \text{ g kg}^{-1} \text{ DM}$ with a wide variability (CV=43.1%) and this could have
8 affected the aerobic stability of silages and consequently the spore content. Average
9 corn silage pH was 3.96 ± 0.18 and the lactic acid content was $48.2 \pm 13.6 \text{ g kg}^{-1} \text{ DM}$
10 whilst no significant levels of butyric acid were detected.
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16 Chemical composition, NDFD and nutritive value of grass, Italian ryegrass, alfalfa hays
17 and silages are reported in table 1. Silages had higher ash and CP contents ($\text{g kg}^{-1} \text{ DM}$)
18 than hays (ash: 71.3 vs 112, $P < 0.001$ and CP: 101 vs 129, $P = 0.05$, for hay and silage
19 respectively) whilst hays had higher NDF content ($557 \text{ vs } 496 \text{ g kg}^{-1} \text{ DM}$, $P = 0.007$,
20 respectively for hays and silages). Silages were also characterized by higher NDFD
21 values than hays ($394 \text{ vs } 476 \text{ g kg}^{-1} \text{ DM}$, $P = 0.002$ for hay and silage, respectively).
22 These differences result in a higher organic matter digestibility (OMd) and net energy
23 for lactation (NE_l) for silages than hays (OMd = $702 \text{ vs } 642 \text{ g kg}^{-1} \text{ DM}$, $P = 0.01$, for
24 silage and hay, respectively; $\text{NE}_l = 4.86 \text{ vs } 4.26 \text{ MJ kg}^{-1} \text{ DM}$, $P = 0.02$; for silage and hay,
25 respectively). Overall, the mean values were low showing a poor quality of the forages
26 used in the TMR.
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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

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6 present study, ASFB tended to be higher for silages (alfalfa, Italian ryegrass and grass)
7 than hays (3.22 vs 2.85 log₁₀ MPN g⁻¹ for silage and hay samples, respectively, P=0.09)
8 but differences were not detected among forages. Grass species silages however had a
9 higher ASFB spore content than corn silage (3.22 vs 2.75 log₁₀ MPN g⁻¹ respectively).
10 Concerning alfalfa silage, the average ASFB (3.00 log₁₀ MPN g⁻¹) was higher than the
11 value (2.11) reported by Colombari *et al.*⁹ for a sample with a similar DM. On average,
12 alfalfa silages had a very high pH value (6.03) and a low lactic acid concentration (9.8 g
13 kg⁻¹ DM). Similarly, contents of ASFB in silages were not in the range of those reported
14 for grass chopped herbage and for well-conserved haylage.^{2,31,32} Furthermore,
15 differently from what reported by Julien *et al.*² clostridia were detected in hay samples.
16 Information about concentrations of butyric acid bacteria spores in farm-scale silages is
17 rather scarce. Studies in France conducted in the 1970s showed that about 20% of grass
18 silages contained more than 10⁵ butyric acid bacteria spores, indicating a poor quality of
19 forages⁸ and in agreement with the results of the present study.
20 Overall, average ASFB content for TMR was 4.75 ±0.73 log₁₀ MPN g⁻¹ and higher than
21 the values estimated from the ASFB spore content of the forages.
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40 **Effect of silage quality and management practices on anaerobic spore** 41 **contamination of milk**

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43 The ASFB content in bulk tank milk of farms that used other ensiled forages in the diet,
44 in addition to corn silage (2.73 log₁₀ MPN L⁻¹), did not differ much from the content of
45 farms that used only corn silage (2.78 log₁₀ MPN L⁻¹) (Table 2). This result was
46 probably due to the small quantity of other silages used in the diet (105 g kg⁻¹ DM on
47 average); the results of the present experiment are consistent with a study of Vissers *et*
48 *al.*¹ which demonstrated that on Dutch farms, corn silage was a more important source
49 of ASFB contamination in milk than grass silage.
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6 Table 2 shows farm distribution and ASFB values in bulk tank milk considering corn
7 silages quality. Farms that used high quality corn silage (lactic acid $>50 \text{ g kg}^{-1} \text{ DM}$,
8 acetic acid $>20 \text{ g kg}^{-1} \text{ DM}$, propionic acid $<1.5 \text{ g kg}^{-1} \text{ DM}$) had a lower spore count in
9 bulk tank milk than the others farms. The concentration of propionic acid in the corn
10 silage seems to influence more the bacterial contamination of milk: farms with low
11 propionic acid content in the corn silage had 13.8% less ASFB in milk than the other
12 farms. All corn silage pH values were below the threshold of 4.2, which can indicate
13 fermentative problems of the silage. High silage pH could be due to: high dry matter at
14 ensiling ($> 50\% \text{ DM}$), cold weather during harvest, slow or poor packing, silage with
15 excess ammonia or urea, clostridial contamination, spoiled or moldy silages, silages
16 containing manure.³³

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28 Farms characterized by lower ASFB content in corn silage and TMR produced milk
29 with lower level of anaerobic spores than the other farms (table 2). A linear regression
30 analysis indicated that there was a positive relationship between milk ASFB ($\log_{10} \text{ MPN}$
31 L^{-1}) and corn silage ASFB ($\log_{10} \text{ MPN g}^{-1}$) content ($y= 0.294*\text{ASFB corn silage} +$
32 2.075 ; $r^2=0.217$). It is well known that high concentrations of anaerobic spores are
33 associated with anaerobic instability of silage during the primary fermentation phase.⁸
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39 However, as recently demonstrated by Vissers *et al.*¹ milk spore contamination is
40 mainly related to aerobic instability rather than to anaerobic instability problems;
41 therefore, also corn silage, being subjected to aerobic deterioration, has an important
42 effect on milk spore contamination.

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

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6 complete milking routine operations (use of gloves, dry udder clean, forestripping,
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8 predipping, postdipping), achieved the lowest ASFB milk contamination. These results
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10 are consistent with those of Zucali *et al.*³⁴ who found that the dairy farms which used an
11
12 accurate milking routine produced milk with the best microbiological quality in terms
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14 of standard plate count, propionibacteria and coliform count.

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

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22 Table 4 shows the frequency of different *Clostridium* species in the forages and in the
23
24 TMR collected in the study. The frequency of *C. tyrobutyricum* was the highest, in
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26 particular in corn silage (66.7%), grass hay (77.8%) and TMR samples (82.6%). *C.*
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28 *beijerinckii* was the second species for presence in feeds, particularly in TMR samples
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30 (47.5%). The almost constant detection of *C. tyrobutyricum* in the sampled forages
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32 arises some concern because, as demonstrated by Klijn *et al.*³⁵, late blowing in Gouda
33
34 cheese was exclusively associated with the growth of *C. tyrobutyricum* and not with the
35
36 presence of *C. beijerinckii* or *C. sporogenes*. In five samples where ASFB were present,
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38 none of the tested clostridia was detected. The presence of other Clostridia can be
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40 hypothesized; for example Rossi and Dellaglio³⁶ detected *C. saccharolyticum* and *C.*
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42 *baratii* in silages whilst Julien *et al.*² identified *C. disporicum* as another predominant
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44 member of clostridial population in silage. Clostridial species present in forages were
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46 consistent with milk spore contamination, confirming the strict relationship.

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48 All the tested species were detected in milk samples.

49
50 Figure 1 shows the results of multiple correlations between variables. The
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52 correspondence analysis divided the variables in four groups. The groups identified
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54 different farm situations based on management practices and milk and feeds quality,
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56 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 log₁₀ 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 log₁₀ MPN g⁻¹, <4.7 log₁₀ MPN g⁻¹).

Milk and cheese quality

The ASFB content in milk was 3.14 and 2.33 log₁₀ 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 log₁₀ 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 halves: in one half lysozyme was added into the vat ($25 \text{ mg } \mu\text{L}^{-1}$) while no addition was done in the other one. After 7 months of ripening, even if spore count was below the analytical limit ($<30 \text{ MPN g}^{-1}$),

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 ($107 \text{ g kg}^{-1} \text{ DM}$ 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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REFERENCES

1. Vissers MMM, Driehuis F, Te Giffel MC, De Jong P and Lankveld JMG, Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration. *J Dairy Sci* **90**:928–936 (2007).
2. Julien MC, Dion P, Lafreniere C, Antoun H and Drouin P, Sources of [Clostridia clostridia](#) in raw milk on farms. *Appl Environ Microbiol* **74**:20 6348–6357 (2008).
3. Garde S, Arias R, Gaya P and Nuñez M, Occurrence of *Clostridium* spp. in ovine milk and Manchego cheese with late blowing defect: identification and characterization of isolates. *Int Dairy J* **21**:272–278 (2011).
4. Borreani G and Tabacco E, Low permeability to oxygen of a new barrier film prevents butyric acid bacteria spore formation in farm corn silage. *J Dairy Sci* **91**:4272–4281 (2008).
5. Neviani E, Bottari B, Lazzi C and Gatti M, New developments in the study of the microbiota of raw-milk, long-ripened cheeses by molecular methods: the case of Grana Padano and Parmigiano Reggiano. *Front Microbiol* **4**:1-14 (2013).
6. IZSLER, Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna “Bruno Ubertini” <http://www.izsler.it/> (January 2014).
7. Vissers MMM, Driehuis F, te Giffel MC, De Jong P and Lankveld JMG, Minimizing the level of butyric acid bacteria spores in farm tank milk. *J Dairy Sci* **90**:3278–3285 (2006).
8. Driehuis F, Silage and the safety and quality of dairy foods: a review. *Agric Food Sci* **22**:16-34 (2013).

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6 9. Colombari G, Borreani G and Crovetto GM, Effect of ensiling alfalfa at low and
7 high dry matter on production of milk used to make Grana cheese. *J Dairy Sci*
8 **84**:2494–2502 (2001).
9
- 10 10. Jonsson A, The role of yeasts and clostridia in silage deterioration. Ph.D. Diss.
11 Swedish University of Agricultural Sciences, Uppsala, Sweden (1989).
12
- 13 11. McDonald P, Henderson AR and Heron SJE, The biochemistry of silage. 2nd
14 edition. Chalcombe Publications, Marlow, Bucks, UK (1991).
15
- 16 | 12. Colombari G, Borreani G and Crovetto GM, Comparison of ~~Lucerne~~-lucerne silage
17 and ventilated hay in maize silage-based rations for dairy cows for the production of
18 milk destined for Grana cheese. *Grass Forage Sci* **54**:184–194 (1999).
19
- 20 | 13. Nadeau E, Arnesson A and Bengtsson A, Investigation of ~~Clostridial~~-clostridial
21 spores in Swedish dairy herds. 14th International Symposium Forage Conservation
22 Brno, Czech Republic, pp 80-82 (2010).
23
- 24 14. Rasmussen MD, Galton DM and Petersson LG, Effects of premilking teat
25 preparation on spores of anaerobes bacteria, and iodine residues in milk. *J Dairy Sci*
26 **74**:2472-2478 (1991).
27
- 28 15. Arias C, Oliete B, Seseña S, Jimenez L, Pérez-Guzmán MD and Arias R,
29 Importance of on-farm management practices on lactate-fermenting *Clostridium*
30 spp. spore contamination of Manchega ewe milk: determination of risk factors and
31 characterization of *Clostridium* population. *Small Rum Res*, **111**:120-128 (2013).
32
- 33 16. AOAC, Official Methods of Analysis, 16th edn. Association of Official Analytical
34 Chemists, Washington, DC, USA (1995).
35
- 36 17. AOAC, Official Methods of Analysis, 17th edn. Association of Official Analytical
37 Chemists, Washington, DC, USA (2000).
38
- 39 18. Mertens DR, Gravimetric determination of amylase-treated neutral detergent fiber
40 in feeds with refluxing in beakers or crucibles: collaborative study. *J AOAC Int*
41 **85**:1217-1240 (2002).
42
- 43 19. Van Soest PJ, Robertson JB and Lewis BA, Methods of dietary fiber, neutral
44 detergent fiber and non-polysaccharides in relation to animal nutrition. *J Dairy Sci*
45 **74**:3583-3597 (1991).
46
- 47 20. Canale A, Valente ME and Ciotti A, Determination of volatile carboxylic acids
48 (C1–C5i) and lactic acid in aqueous acid extracts of silage by high performance
49 liquid chromatography. *J Sci Food Agr* **35**:1178-1182 (1984).
50
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6 21. Spanghero M, Berzaghi P, Fortina R, Masoero F, Rapetti L, Zanfi C, *et al*, Precision
7 and accuracy of in vitro digestion of neutral detergent fiber and predicted net energy
8 of lactation content of fibrous feeds. *J Dairy Sci* **93**:4855-4859 (2010).
9
- 10
11 22. NRC, Nutrient Requirement of Dairy Cattle, 7th edn. National Research Council,
12 National Academy of Science, Washington, DC, USA (2001).
13
- 14 23. Cremonesi P, Vanoni L, Silvetti T, Morandi S and Brasca M, Identification of
15 *Clostridium beijerinckii*, *Cl. butyricum*, *Cl. sporogens*, *Cl. tyrobutyricum* isolated
16 from silage, raw milk and hard cheese by a multiplex PCR assay. *J Dairy Res*
17 **79**:318-323 (2012).
18
- 19 24. Carini S and Casadei S, Contributo alla conoscenza della respirazione e dei
20 metabolismi ossidativi nei propionici, *Sci Tec Latt Cas* **21**:365-378 (1970).
21
- 22 25. Schreiner DA and Ruegg PL, Relationship between udder and leg hygiene scores
23 and subclinical mastitis. *J Dairy Sci* **86**:3460-3465 (2003).
24
- 25 26. SAS 9.2. SAS Inst. Inc., Cary, NC (2009).
26
- 27 27. te Giffel MC, Wagendorp A, Herrewegh A and Driehuis F, Bacterial spores in
28 silage and raw milk. *A Van Leeuw J Microbiol* **81**:625-630 (2002).
29
- 30 28. Borreani G and Tabacco E, The relationship of silage temperature with the
31 microbiological status of the face of corn silage bunkers. *J Dairy Sci* **93**:2620-2629
32 (2010).
33
- 34 29. Danner H, Holzer M, Mayrhuber E and Braun R, Acetic acid increases stability of
35 silage under aerobic conditions. *Appl Environ Microbiol* **69**:562-567 (2003).
36
- 37 30. Muck RE, Moser LE and Pitt RE, Postharvest factors affecting ensiling, pp 251-304.
38 In *Silage Science and Technology*. Buxton DR, Muck RE and Harrison JH ed. Am.
39 Soc. Agron., Crop Sci. Soc. Am., Soil Sci. Soc. Am., Madison, WI (2003).
40
- 41 31. Rammer C, Quality of grass silage infected with spores of *Clostridium*
42 *tyrobutyricum*. *Grass Forage Sci* **51**:88-95 (1996).
43
- 44 32. Müller CE, Fermentation patterns of small-bale silage and haylage produced as a
45 feed for horses. *Grass Forage Sci*. **60**:109-118 (2005).
46
- 47 33. Kung L and Shaver R, Interpretation and use of silage fermentation analysis reports.
48 *Focus on Forage* **3**:13 1-5 (2001).
49
- 50 34. Zucali M, Bava L, Tamburini A, Brasca M, Vanoni L and Sandrucci A, Effects of
51 season, milking routine and cow cleanliness on bacterial and somatic cell counts of
52 bulk tank milk. *J Dairy Res* **78**:436-441 (2011).
53
- 54 35. Klijn N, Nieuwenhof FFJ, Hoolwerf JD, van der Waals CB and Weerkamp AH,
55 Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in
56
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5
6 cheese by species-specific PCR amplification. *Appl Environ Microbiol* **61**:2919–
7 2924 (1995).
8

9
10 36. Rossi F and Dellaglio F, Quality of silages from Italian farms as attested by number
11 and identity of microbial indicators. *J Appl Microbiol* **103**:1707-1715 (2007).
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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 ₁ 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; NE₁ 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

^eSE: standard error

^fP: 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
<i>Farms size</i>			
lactating cows < 100	12	2.78	0.60
lactating cows > 100	11	2.72	0.45
<i>Grass and alfalfa silage</i>			
NO	11	2.78	0.37
YES	12	2.73	0.65
<i>Corn silage quality</i>			
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
<i>TMR^d spore count</i>			
\log_{10} MPN g^{-1} < 4.7	10	2.61	0.57
\log_{10} MPN g^{-1} > 4.7	13	2.86	0.48
<i>Corn silage spore count</i>			
\log_{10} MPN g^{-1} < 2.3	10	2.49	0.53
\log_{10} MPN g^{-1} > 2.3	11	3.00	0.40

^aMPN: Most probable number

^bSD: standard deviation

^cDM: dry matter

^dTMR: 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

	No. farms	Anaerobic spore forming bacteria count	
		Mean	SD ^b
<i>Udder hygiene</i>			
dirty udder < 40% ^c	13	2.58	0.49
dirty udder > 40%	10	2.98	0.51
<i>Milking routine</i>			
use of gloves: NO	12	2.81	0.51
use of gloves: YES	11	2.7	0.56
<i>Dry udder clean</i>			
NO	7	2.73	0.36
YES	16	2.76	0.6
<i>Forestripping</i>			
NO	9	2.92	0.51
YES	14	2.65	0.53
<i>Predipping</i>			
NO	14	2.79	0.49
YES	9	2.69	0.6
<i>Postdipping</i>			
NO	8	2.76	0.58
YES	15	2.75	0.52
<i>Milking routine: no. operations</i>			
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

^aMPN: most probable number

^bSD: standard deviation

^cDirty udder: % of udder with hygiene score 3 and 4

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

		<i>Cl. beijerinckii</i>	<i>Cl. butyricum</i>	<i>Cl. sporogenes</i>	<i>Cl. tyrobutyricum</i>
	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
<i>Milk production</i>				
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
<i>TMR composition</i>				
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
<i>Forages spore contamination</i>				
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
<i>Hygiene Score</i>				
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
<i>Milk bacterial counts</i>				
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

^aSE: standard error

^bP: probability

^cDMI: dry matter intake

^dMPN: most probable number

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^eSPC: standard plate count
^fCFU: colony forming unit

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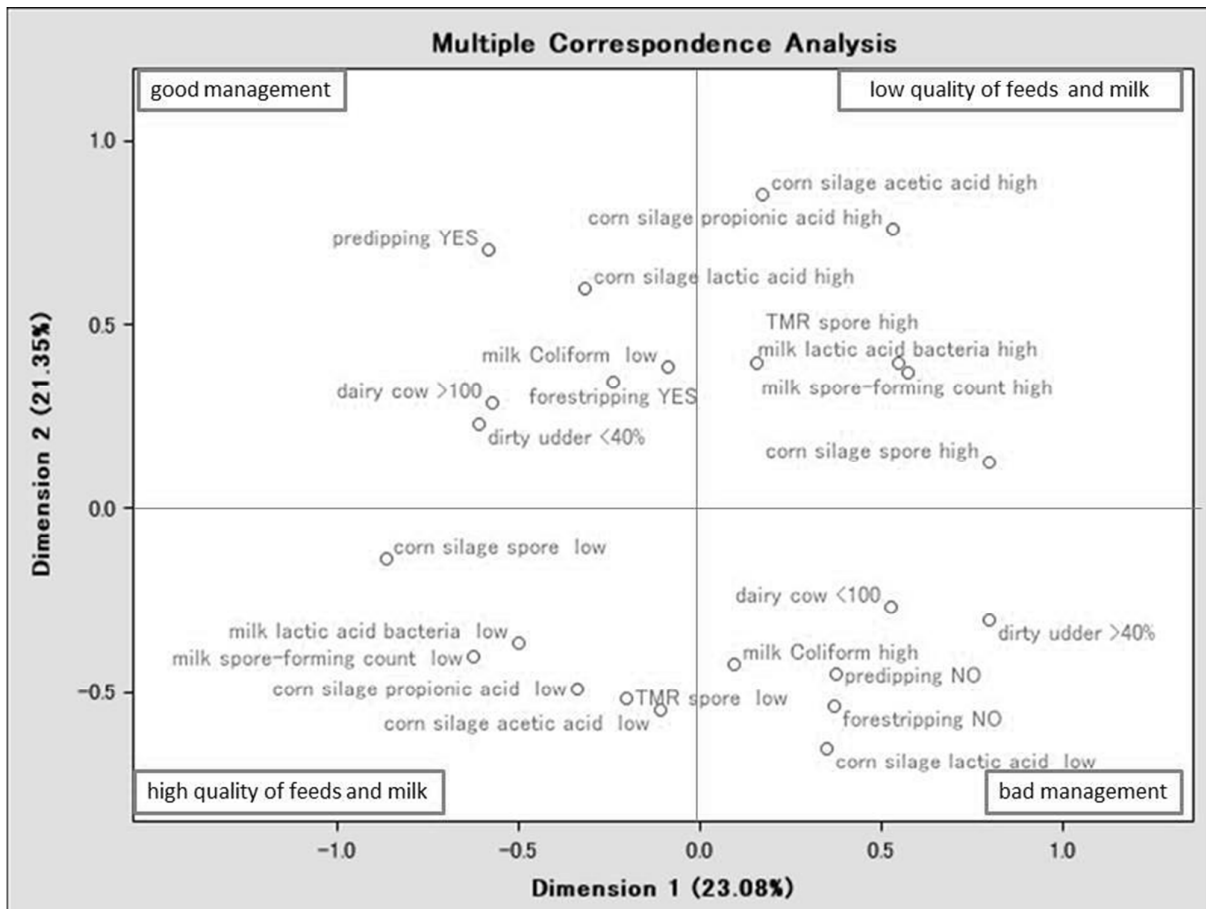


Figure 1. Multiple correspondence analysis of risk factors for spore contamination in milk of 23 farms

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

