dairy

MDPI

Article

Farm Silage Facilities and Their Management for the Prevention of Anaerobic Bacteria Spore Contamination in Raw Milk

Fabio Abeni [1,*], Rosanna Marino [1], Francesca Petrera [1], Giulia Segati [1], Andrea Galli [1,2] and Domenico Carminati [1]

[1] Centro di Ricerca Zootecnia e Acquacoltura, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), via Lombardo 11, 26900 Lodi, Italy; rosanna.marino@crea.gov.it (R.M.); francesca.petrera@crea.gov.it (F.P.); giulia.segati18@gmail.com (G.S.); a.galli@aral.lom.it (A.G.); domenico.carminati@crea.gov.it (D.C.)

[2] Associazione Regionale Allevatori Lombardia (ARAL), via Kennedy 30, 26013 Crema, Italy

* Correspondence: fabiopalmiro.abeni@crea.gov.it

Abstract: At feed-out, aerobic spoilage of silage enables an increase in anaerobic spore-forming bacteria (ANSB) that may enter the total mixed ration (TMR). The aim of our study was to understand whether in hot summers the silage structures and management may affect the level of ANSB in milk for long-ripening cheese production. A survey of silage facilities, management, and their relationships with silage, TMR, feces, and milk ANSB most probable number (MPN) content was conducted in the Po Valley during summer months. Silo type did not affect the mean ANSB, but only the wideness of their value distributions, with a narrow range for bags and a wider range for bunkers. The unloading equipment affected the ANSB count; the front-end loader with cutter was associated with a lower ANSB count—probably as a result of the reduced surface left after daily silage removal. Silo length and daily removed face width were the main factors affecting contamination of silage by spore-forming bacteria during summer, with longer silos and wider surface removal reducing ANSB contamination—probably as a consequence of reduced aerobic spoilage at the silage surface. The silage contamination by spore-forming bacteria within a $\log_{10}$ 2 MPN g^{-1} allowed a low concentration of spore-forming bacteria at the farm bulk milk tank level. Fecal ANSB levels did not factor into the regression that explains the ANSB in farm milk. It has been found that silage facilities' features and their management are an important first step to reduce the extent of ANSB contamination at the farm level.

Keywords: aerobic spoilage; cheese defect; corn silage; spore-forming bacteria

Citation: Abeni, F.; Marino, R.; Petrera, F.; Segati, G.; Galli, A.; Carminati, D. Farm Silage Facilities and Their Management for the Prevention of Anaerobic Bacteria Spore Contamination in Raw Milk. *Dairy* **2021**, *2*, 500–514. https://doi.org/10.3390/dairy2030040

Academic Editor: F. Javier Giráldez

Received: 21 June 2021
Accepted: 5 September 2021
Published: 16 September 2021

1. Introduction

Corn for silage is the most frequently cultivated crop in Lombardy on the left part of the Po Valley area—the most important milk production area in Italy—mainly used for the manufacturing of typical Italian long-ripening protected designation of origin (PDO) cheeses—chief among which is the Grana Padano cheese, because of its easy conservation by ensiling [1]. Systems based on corn silage as the main forage source for dairy cows, in these conditions, allow the highest income over feed cost (IOFC) when compared to the other possible forage systems for this environment [2]. The massive presence of corn silage—frequently associated with other ensiled forages—represents a critical control point for the production chain of a long-ripening cheese, because it is the main contamination source of anaerobic spore-forming bacteria (ANSB) [3]. The ANSB are of major interest in long-ripening cheese production because they are responsible for the late-blowing defect [4]. Among the different species, *Clostridium tyrobutyricum* is considered the most important bacteria responsible for this problem [5]. The contamination chain was well summarized by Doyle et al. [6]: from silage to the total mixed ration (TMR); from TMR to feces; from feces to the cow udder and milking unit. However, the extent of this

contamination varies across seasons and years [7,8]. Miller et al. [9] reported the central role of different management practices in limiting the contamination of milk by mesophilic and thermophilic spore formers; most such practices fall under the umbrella of milking hygiene management [10,11]. These practices may be effective only at the last step of the farm's contamination chain.

Bermúdez et al. [7] reported a seasonal distribution of ANSB ($\log_{10}$ MPN/L) in bulk milk with two peaks: one in winter and one in summer; they also showed that *Clostridium tyrobutyricum* is the prevalent species isolated in milk samples (always above 50% of spore-forming bacteria) throughout the year. This species is well known as the main causative agent of late-blowing defects in ripened cheese [6]. In Lombardy (Italy), the laboratory for regional milk quality payment [8] recorded a decrease in ANSB bulk milk contamination in 2019, and a less evident trend in the contamination by spore-forming bacteria in the summer and autumn months, compared to 2017–2018. A partial explanation of this seasonal pattern arises from the change in gut physiology in dairy cows under summer heat stress [12]. However, a possible further contribution to this seasonal pattern in spore-forming bacteria may be also related to changes in feed's microbiological quality, particularly for silage ingredients.

Vissers et al. [3] reported that silages—and especially corn silage—are the most important sources of variation affecting the possible contamination chain of ANSB; therefore, a seasonal dynamic for this feed must be considered. Storm et al. [13] reported a seasonal dynamic in corn silage microbiota; however, in their environmental situation (Denmark), the temperature was not a driving factor—probably because it did not reach temperature/humidity conditions comparable to those reported in the Po valley by Calamari et al. [12].

Recently, Borreani et al. [14] focused their attention on the role of silages and their management (namely, in the feed-out phase) to explain the most probable sources of spore contamination in raw milk at the farm level. Zucali et al. [4] observed that high spore content TMRs may include starting ingredients (silages) with an average ANSB contamination lower than that of the mix. The hypothesis of a multiplication during the hours after diet mixing was explored by the model of Vissers et al. [3], but the possible simulations do not explain the gap between traditionally sampled silages and the level of TMR contamination. Vissers et al. [15] and Borreani et al. [14] suggested that this gap could be explained by the role of the more spoiled parts of some silage that is not correctly and fully removed at feed-out before the daily diet preparation.

Bernardes et al. [16] reported that high temperatures at the time of unloading can affect the growth rates of spoilage microbes; their review underlined how the optimal growth of most yeast species occurs at 30 °C—an environmental temperature that in Italy is increasingly recorded in spring (and obviously in summer) owing to climate change. This was already reported by Ashbell et al. [17] with corn and wheat silage. Bernardes et al. [16] indicated the range for the optimal growth of molds and clostridia as falling between 25 and 37 °C. It is for these reasons that the present study concentrated all of its efforts on the hot season. In these situations, two main points were suggested to be crucial for the prevention of spoilage at silage feed-out: the daily removal rate (due to the daily inclusion of silage in the diet), and the silo dimensions [18].

A central point in silage daily feed-out rate is its management, in terms of depth and width of face removed, as well as in terms of the defacer technology used. In their study, Heguy et al. [19] reported that a large share of farmers used a front-end loader (85.1%), but some dairies used a rake (10.8%) or a defacer (4.1%). Silo dimensioning is a choice that sets up the constraints within which the farmer will manage silage feed-out in the long term. Silos are an important investment within dairy farms; the type and size of silo represent important factors affecting silo price and, therefore, the silage production cost in dairy farms [20].

The aim of our study was to understand whether the silage structures and management during summer heat may affect the level of spore-forming bacteria in milk for long-ripening cheese production.

2. Materials and Methods

2.1. Farm Characteristics

A total of 108 dairy farms were chosen from the farms that produce raw milk for the manufacturing of Grana Padano PDO cheese in the Province of Cremona (Lombardy Region, northern Italy). Participants were selected based on the following criteria: willingness to participate in the face-to-face survey and to be monitored for microbiological analysis of the feedstuffs, feces, and milk of their herds; high use of silage in the diets of lactating cows, with whole corn as the main silage in terms of supplied dry matter. The prevailing cattle breed in the monitored farms was the Italian Frisian. Milking was carried out twice daily via mechanical milking systems in all of the farms. The farmers were fully informed of the aims and design of the trial, as well as about the possible use of the collected data.

The technicians of the Regional Breeders Association of Lombardy (ARAL) visited each dairy farm once during the summer of 2018 to collect all of the data about herd and silage management. A questionnaire was filled out to collect information on silage production and management (see Table 1).

Table 1. Main points of the questionnaire adopted in the survey on silo features and management in the 108 dairy farms in Lombardy.

Information	Description
General	ID code; location; dairy processor
Silo type	Bunker; pile; bag
Building year	(only for bunker)
State of maintenance	1 = bad; 2 = fairly good; 3 = very good (only for bunker)
Dimensions	Length; width; lateral wall height; maximum silage mass height (only for bunker)
Top surface shape	1 = spiked; 2 = rounded; 3 = flat
Forage type	...
Inoculants	0 = absent; 1 = present
Covering material	0 = only polyethylene film: 1 = with oxygen barrier film
Films on lateral walls	0 = absent; 1 = present (only for bunker)
Materials above film	Gravel bags; concrete tiles; stones; other
Unloading system	1 = front-end loader; 2 = front-end loader with cutter; 3 = defacer
Depth of face removed (daily)	1 = < 15 cm d^{-1}; 2 = 15–30 cm d^{-1}; 3 = 30–45 cm d^{-1}; 4 = > 45 cm d^{-1}
Width of face removed (daily)	1 = < 25%; 2 = 33%; 3 = 50%; 4 = 100%
Spoilage type [1]	0 = not spoiled silage; 1 = upper surface of the mass; 2 = lateral border of the mass; 3 = upper lateral corners; 4 = upper surface and several spot within the mass; 5 = like "4", with several horizontal layers within the mass; 6 = spoiled horizontal layer at the bottom of the mass

[1] Partly adapted from Mickan et al. [21].

The different kinds of spoilage and the reference images for the state of bunker silo maintenance are also reported in Figure 1.

2.2. Sample Collection, Preparation, and Microbiological Analyses

During each visit, feces, silages (e.g., corn silage, grass silage, ear-corn silage, etc.), total mixed ration (TMR), and raw milk were sampled. During the months of July and August 2018, the maximum daily temperature was ≥30 °C for 57 out of 62 days, constituting good conditions for yeast proliferation on the silage surface. Samples were collected during sunny days, without rain and mud or puddles around the visited area of each farm involved in the study, in one visit only per farm. All necessary precautions were adopted to avoid dust contamination of different matter as a consequence of tractors' movements in the farms before sampling. Fresh individual feces from a random 10% of lactating cows within each dietary group were collected and pooled by group. The fecal samples were collected just after emission, from the section not in contact with the floor. Then, a farm feces value was obtained; when 2 or more dietary groups were present, the value from each group was weighed according to the contribution of the same group to the farm's daily milk production. The silage samples were collected from different points within the core

area in each silo. According to Gallo et al. [22], 2 kg subsamples (on a wet basis) from each silo were collected from 5 randomly chosen points, at least 30 cm from the lateral silo walls as well as from the upper silo surface; then, they were mixed to obtain a single 2 kg sample for the laboratory analysis. The TMR samples fed to lactating cow groups were collected immediately after unloading (by taking 10 subsamples of ~200 g, pooled by dietary group, to extract a final sample of 1 kg for each group). Milk samples (1 L) were collected in sterile bottles from the farm's bulk tank at the end of the morning milking on the day after the collection of the feed and feces samples.

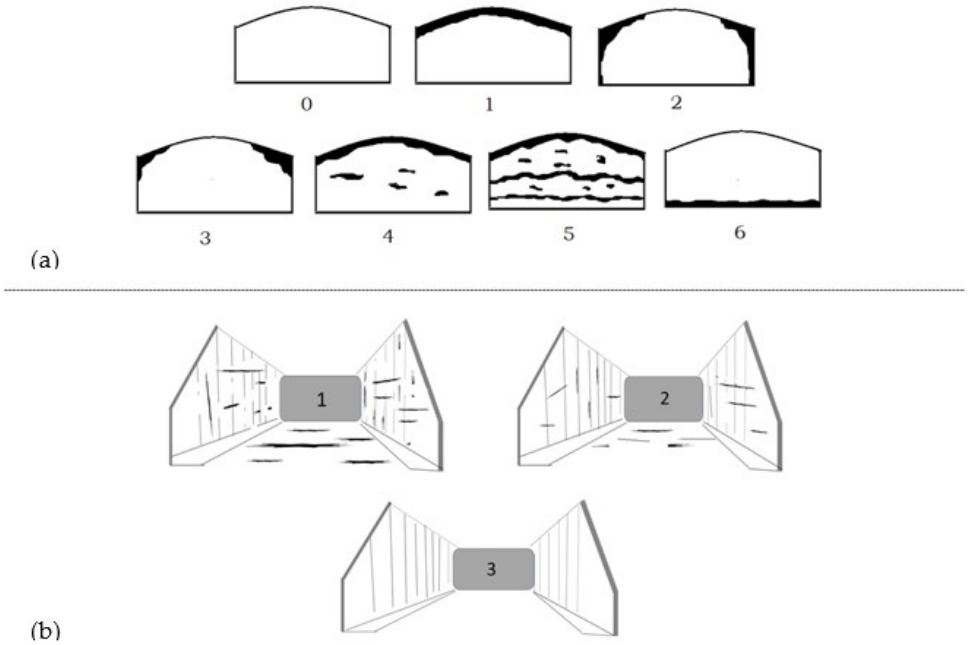

Figure 1. (**a**) Silage visual spoilage code used to identify the distribution of the spoiling point on the silo face (adapted from Mickan et al. [21]); (**b**) visual code to identify the bunker maintenance level.

All samples were transported to the laboratory under refrigeration (4 °C); milk samples were analyzed within 24 h of collection, while the other samples were stored at −20 °C until analysis. Each silage, TMR, feces, and bulk milk tank sample was analyzed for enumeration of gas-producing ANSB.

In feed, feces, and milk samples, the ANSB spore concentration was estimated by the most probable number (MPN) technique. The procedure was performed with a 3 × 5 scheme (three 10-fold dilutions and five tubes for each dilution). One-milliliter aliquots of decimal dilutions were inoculated into each of 5 tubes containing 5 mL of the culture medium based on reconstituted sterile skimmed milk supplemented with a lactate–acetate solution [4,23,24]. The inoculated tubes were then sealed with 2 mL of melted paraffin and heated at 85 °C for 15 min to inactivate vegetative cells and to trigger the germination of spores. Tubes were incubated at 37 °C for 7 d. A positive result (i.e., gas production) was indicated by a completely lifted paraffin plug. The combined number of gas-positive tubes in the last three serial dilutions was used to evaluate the number of spores. MPN counts and their parameters were estimated using a freely available Excel spreadsheet developed by Jarvis et al. [25], and the result was expressed according to the starting matrix amount (spore MPN g^{-1} or l^{-1}).

2.3. Statistical Analysis

Descriptive statistics were calculated for all of the variables (structure as well as management) recorded for each silo.

The MPN data of ANSB counts were $\log_{10}$-transformed (MPNld) for statistical analysis. For MPN results, spore counts below the detection limit (i.e., 36 spores/g in silage, TMR,

and feces, or 360 spores/L in milk) were assigned a value corresponding to half of the detection limit to calculate the average value. For MPN results above the maximum value allowed by the dilutions inoculated, the maximum values were used.

All statistical analyses were performed in R [26]. For silo data, Pearson's correlations were calculated between the MPNld and the silos' structural and management features. Linear regression analysis was performed to explain possible relationships between the MPNld and the structural and management features of each silo, and select the best available model.

For farm data, Pearson's correlations were calculated between the MPNld in corn silage, TMR, feces, and milk samples collected during the same visit. A linear regression analysis was performed to assess—considering the spore levels in silage, TMR, and feces—the model that best explains the spore levels in milk. All of the possible combinations of these independent variables (including their quadratic effects) were considered, and the best was retained according to the Akaike information criterion. A diagnostic step after each regression analysis was performed by checking the residual distribution.

When important practical, categorical, or class-transformed quantitative features needed to be assessed for a significant effect on silo MPNld values, ANOVA with Tukey's honestly significant difference test, when appropriate, was performed for the pairwise comparisons between least square means according to a linear model by the "lm" procedure in R and using the "lsmean" package. The alpha level for significance was set at 0.05. The different distributions of ANSB between matrices and survey items were represented by violin plots showing the kernel probability density of the data at different values, and including a marker (white dot) for the median of the data and a black box indicating the interquartile range.

3. Results

3.1. Anaerobic Spore-Forming Bacteria Determined in Different Matrices

Table 2 reports the main recorded procedures for each silo and the descriptive statistics for bunker silo dimensions.

The different distributions of anaerobic spore-forming bacteria determined in the different matrices sampled at farm level are reported in Figure 2.

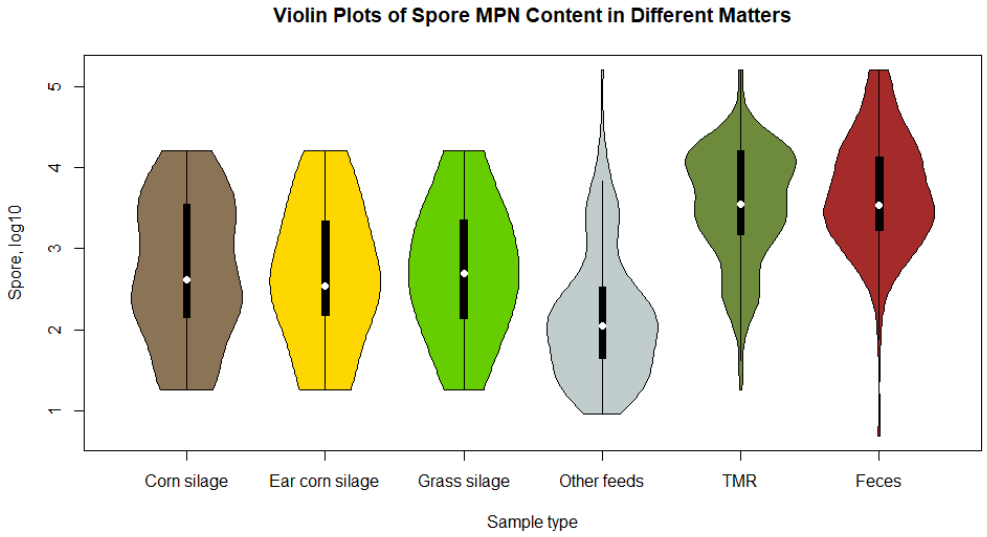

Figure 2. Distribution of anaerobic spore-forming bacteria spores (MPN, $\log_{10} n/g$) for the main silages included in the TMR and for the remaining feeds, for TMR, and for feces collected within the study.

The three most common feeds in the surveyed farms (corn silage, ear-corn silage, and grass silage including winter cereal silage) were compared with other farm-produced feeds

(essentially hays) and with TMR and feces samples by violin plot to stress the different kinds of frequency distributions. The silages had higher ANSB counts than the other farm-produced feeds, but the resulting TMR had higher values, with a left-skewed distribution (−0.6745931). At the same time, fecal samples reported a right-skewed value distribution.

Table 2. Main ensiling procedures recorded for each silo (n = 173).

Ensiling Procedures (All)	Overall
Type of silage storage	
Bunkers	80.3%
Piles	13.9%
Bags	5.8%
Silage additives	
No	57.3%
Yes	42.7%
Type of material placed on the top of plastic film [1]	
None	
Gravel bags	63.5%
Other materials	45.8%
Unloading system	
Front-end loader without cutter	12.6%
Front-end loader with cutter	11.3%
Defacer	76.2%
Type of longitudinal plastic film	
Single	16.7%
Double (with oxygen-barrier)	83.3%
Bunker	
Bunker dimensions	
Width, m	10.36 ± 4.49
Length, m	43.58 ± 19.69
Wall height, m	2.83 ± 0.66
Face surface, m^2	30.48 ± 17.24
Volume, m^3	1372 ± 1133
Lateral plastic film	
Absence	34.5%
Presence	65.5%

[1] The sum is not 100% accurate because some silos may use 2 different kinds of material at the same time.

3.2. All of the Silos

A comparison between the three main types of silo did not find evidence of a significant effect on spore content (Figure 3).

We can see that the distribution of spore values within bag silo was narrower than those in bunker and pile silos. Table 3 shows linear regression analysis results that describe the relationships between silage ANSB and the structural and management features of each silo.

The linear coefficients for "Silo length" and "Width of daily face removal" were negatively associated with the silage MPNld.

Figure 4 shows the distribution of ANSB data from silos with different daily removal width rates.

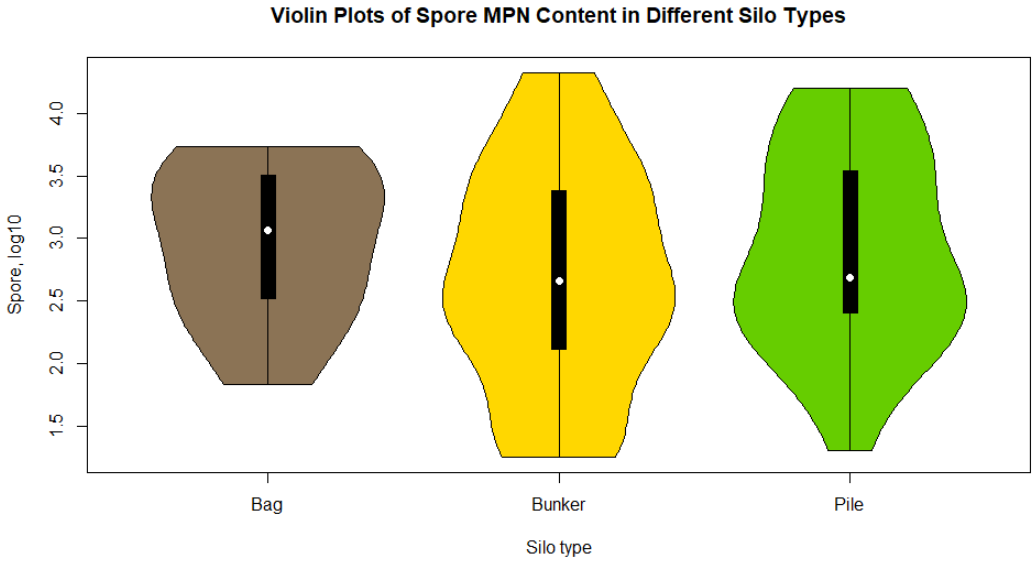

Figure 3. Distribution of anaerobic spore-forming bacteria spores (MPN, $\log_{10}$ n/g) for the three kinds of silo sampled within the study.

Table 3. Linear regression analysis of the log10 most probable number of spores (MPNld) from each silo, and its structural and management features.

Coefficients	Estimate	Standard Error	t Value	*p*
(Intercept)	3.5450000	0.3062000	11.575	<0.001
Silo length, cm	−0.0000915	0.0000448	−2.042	0.0433
Width of daily face removal, class [1]	−0.1577000	0.0765000	−2.061	0.0414
Residual standard error	0.872			
DF	123			
Multiple R-squared	0.06248			
Adjusted R-squared	0.04724			
F-statistic	4.099			0.01891

[1] Classes: 1 = 25% daily removal; 2 = 33% daily removal; 3 = 50% daily removal; 4 = 100% daily removal.

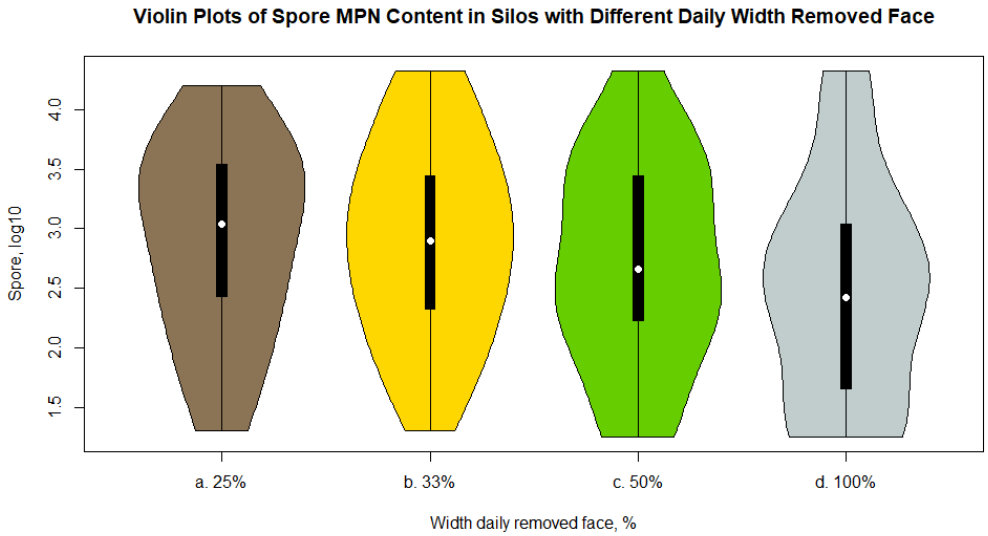

Figure 4. Distribution of anaerobic spore-forming bacteria spores (MPN, $\log_{10}$ n/g) for the four classes of daily removal width of exposed silage surface (percentage) for the silos sampled within the study.

3.3. Silage Unloading Equipment

Figure 5 shows the effect ($p = 0.018$) of unloading system on the silo face spore count. The "front-end loader with cutter" was associated with the best (lower) spore content in the sampled face of the silo that differs ($p = 0.025$) from the values obtained with a "defacer".

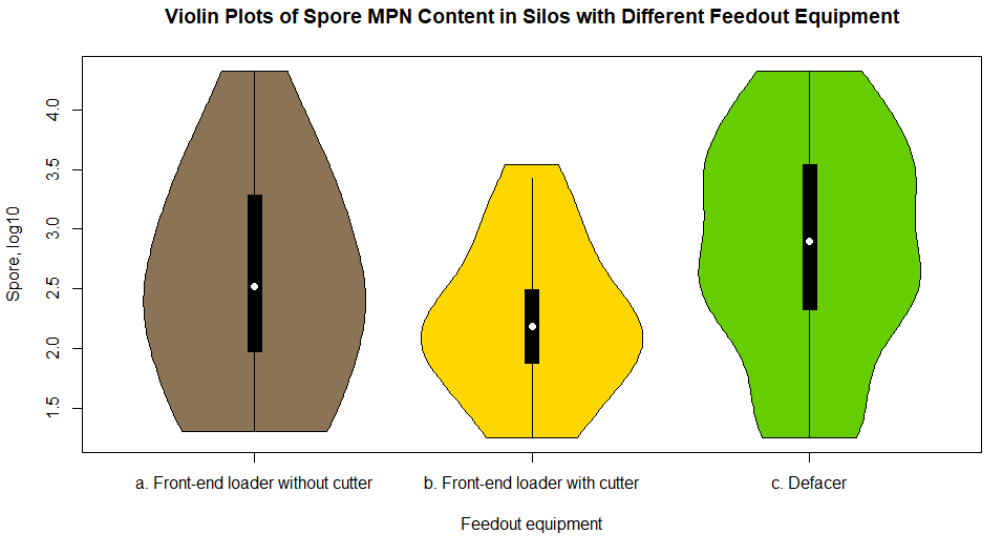

Figure 5. Distribution of anaerobic spore-forming bacteria spores (MPN, $\log_{10}$ n/g) for the three types of feed-out equipment in the silo sampled within the study.

3.4. Bunker Silos

The age class of the bunker silos (based on time from building: <8, 9–16, and >16 years) did not affect ($p = 0.125$) the results for silage ANSB; however, the violin plots in Figure 6 show the different distribution shapes between the three age classes.

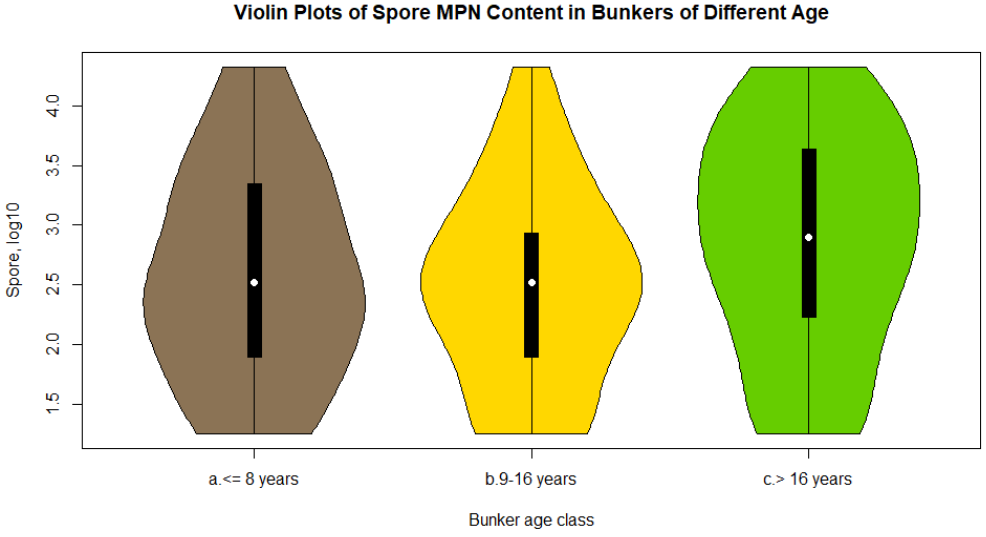

Figure 6. Distribution of anaerobic spore-forming bacteria spores (MPN, $\log_{10}$ n/g) for the three age classes of bunker silos sampled within the study.

A shift in the distribution toward higher values for the older silos was appreciable. The age class of bunker silos did not affect the spore content in our study, even if i the different distribution shapes in Figure 6 seem interesting, where a trend toward higher spore values is appreciable for silos aged more than 16 years.

There was a trend ($p = 0.057$) toward an effect on the kind of spoilage. Based on pairwise comparisons, the spoilage "2" (lateral, Figure 1a) reported higher ($p = 0.040$) spore

values than "1" spoilage (upper surface only). The lack of greater significance is related to the unbalanced frequencies between the different kinds of spoilage, with spoilage "2" less represented than "1" and "3" (see the mosaic representation in Figure 7).

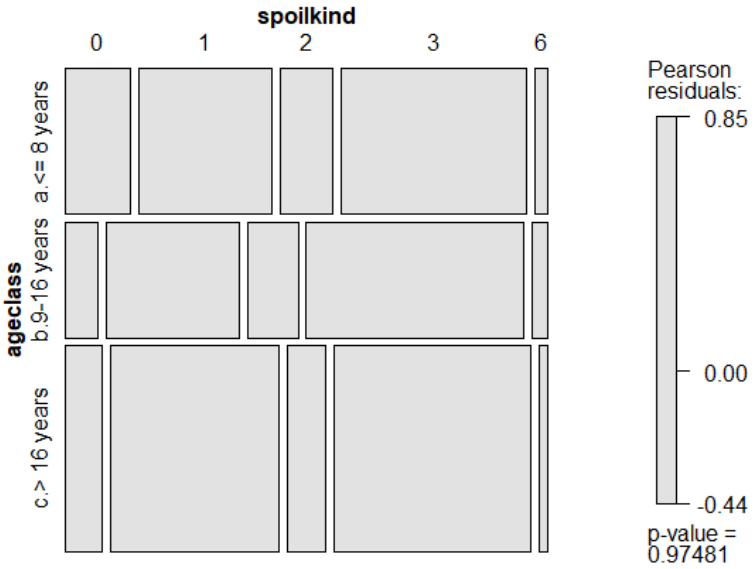

Figure 7. Mosaic graph showing the frequency of different kinds of spoilage in the three classes of bunker age.

3.5. Farms

The multiple linear regression results in Table 4 show the significant coefficient models that relate ANSB in the bulk milk tank in each farm with ANSB counts in both TMR (linear) and corn silage (quadratic).

Table 4. Linear regression analysis of the $\log_{10}$ most probable number of spores (MPNld) from each bulk milk sample, and of the spore content of maize silage and TMR fed to lactating dairy cows from the same herd.

Coefficients	Estimate	Standard Error	t-Value	*p*
(Intercept)	0.26822	0.71387	0.376	0.7083
TMR spore content, $\log_{10}$ MPN g^{-1}	0.20200	0.12298	1.643	0.1052
Maize silage spore content, $\log_{10}$ MPN g^{-1}	0.97769	0.50162	1.949	0.0555
Maize silage spore content, $(\log_{10}$ MPN $g^{-1})^2$	−0.16210	0.09294	−1.744	0.0858
Residual standard error	0.5611			
DF	66			
Multiple R-squared	0.1214			
Adjusted R-squared	0.08151			
F-statistic	3.041			0.03496

No models including fecal ANSB count showed significant results to explain milk ANSB content. Stratifying farms by the level of corn silage spore content in three classes, the ANOVA model evidenced a significant effect of corn silage contamination on farm milk ANSB (model $p = 0.024$), with the lowest MPNld value in the lowest class of corn silage ANSB contamination (2.03 ± 0.13, 2.50 ± 0.11, 2.34 ± 0.12 as LSMEANS and SE for the three classes, respectively, in growing order of ANSB in corn silage). The violin plot in Figure 8 shows the different distributions of MPNld between the three corn silage classes.

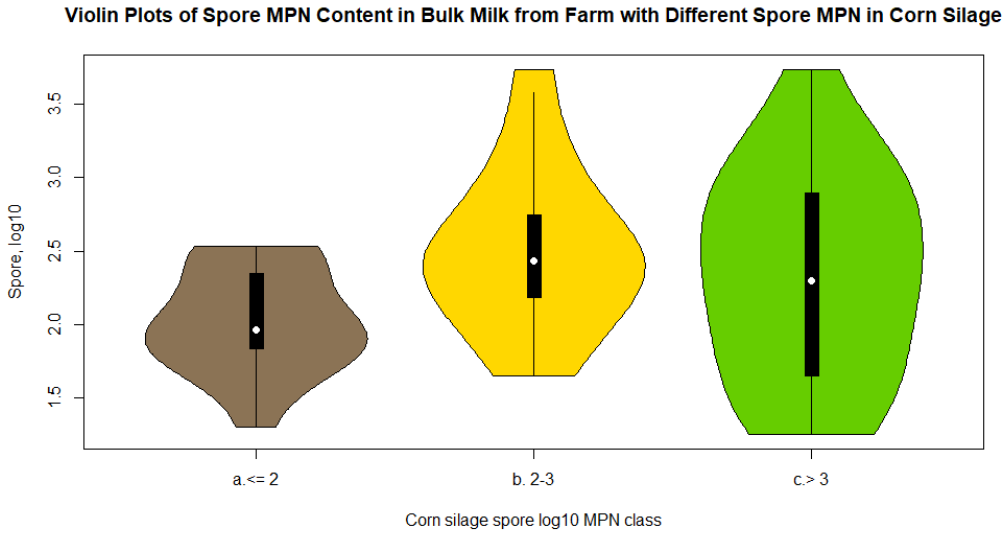

Figure 8. Distribution of anaerobic spore-forming bacteria spores (MPN, $\log_{10}$ n/L) in bulk milk tanks from farms in three different classes of maize silage spore MPN ($\log_{10} n/g$) sampled within the study.

4. Discussion

4.1. Anaerobic Spore-Forming Bacteria in Different Matrices

The ANSB contamination in the dairy chain starts from the forages (in this case, silages), their inclusion in TMR preparation, and their passage through the bovine digestive system (feces), thus determining the risk of milk contamination from the environment. The violin plot in Figure 2 allows us to appreciate the different distribution of MPNld values within each matrix behind its respective mean. The silage sampling was performed to obtain a representative sample of the "assumed" edible portion of the feed, avoiding sampling from visible spoiled silage and, in any case, less than 30 cm distant from the lateral wall or border as well as from the top surface of the silo, similarly to Gallo et al. [22]. In a study on 23 Italian farms using silage for dairy cows that yielded milk destined for Grana Padano PDO cheese production, Zucali et al. [4] reported an overall average ANSB count for TMR (4.75 ± 0.73 MPNld/g) higher than those estimable from the sum of supplies from each silage in the diet; similar evidence was reported by the same researchers in another paper [10]. Recently, Borreani et al. [14] attributed this kind of result to the inclusion of the most critical area of the silo face, not adequately removed before silage unloading. Those critically contaminated parts, if accidentally mixed in the TMR, lead to a very high level of contamination by the spores from the most aerobically spoiled areas—generally those at the top surface or at the top corners of the silo face. This could explain why the good microbiological quality of the silages sampled—avoiding such extreme contamination—appears inconsistent with the results from TMR samples. In the present study, we did not assess the degree of the cleaning of the silo face before TMR preparation, because this was not our aim. However, this result suggests that the common sampling method used to assess contamination by ANSB in silages at the mass core is unreliable for understanding the possible origins of ANSB in TMR. Further, it is clear that, for research studies in particular, the ingesta-to-excreta ratio should not consider the feeds separately, but rather the real TMR at the manger, in order to avoid an underestimation of the ingesta's ANSB count. A specific silage sampling protocol to assess the risk of ANSB contamination from the spoiled parts does not seem realistic because, based on the results of Borreani et al. [14], those parts must be excluded a priori from the TMR; therefore, the TMR itself is a critical control point for the chain.

4.2. All the Silos

The three silo types considered in the present study are the main types used in the Po Valley, with a strong prevalence of bunkers (Table 2). The narrow distribution of spore values within bag silos confirms that this technique is strongly standardized. We must also take into account the fact that the bag type is considered less prone than the other two types to direct contamination during ensiling, since the action of tractor wheels is lacking, thus posing fewer soil contamination risks. Mostafa et al. [27] recently confirmed that a large portion of silage in a bag may be stored at a density below 200–225 kg DM/m^3, which is generally considered an appropriate goal for aerobic spoilage prevention. The comparison with bunker and pile silos can lead to the conclusion that they make it possible to reach a more stable face at air exposure due to higher compaction than in bag, with the minimum value of ANSB being more than 0.5 MPNld/g higher than the minimum attained in the other two silo types (see Figure 3); on the other hand, these two kinds of silo may be subject to extreme negative management that affects the right tail of their distribution frequency.

The contemporary negative relationships of both "Silo length" and "Width of daily face removal" with silage ANSB suggest that the silo layout may affect the susceptibility of the silo face to be prone to high spore-forming bacteria contamination (Table 3). Silo length implicitly suggests that the ensiled mass, which tends to occupy this dimension more than width or height, is subjected to a better feed-out management. At the same time, it is intuitive that the daily removal of the silage surface exposed to air limits the growth of yeasts, thus avoiding aerobic spoilage and consequent restart of clostridia activity [28]. The comparison of MPNld data distribution within each kind of silo reinforces the beneficial effect of this removal, especially in the hot season (Figure 4). The depth of daily silage removal did not affect the spore count in the successive feed-out silage per se; the most probable reason for this is related to the irregular removal of the silo front if the whole area is not removed daily, but only a part of it. In this case, we must consider that the global forage surface exposed to the air increases compared to the least attainable surface due to the total daily removal. In this way, air can also enter from lateral spaces newly exposed by a deeper advancement in the nearest part of the silo, leaving additional surface for the entry of air into the mass. Heguy et al. [19] underlined that some farmers remove a high (good) depth of silage daily, but at the expense of a reduced width (only 1/3 of the front). For these practical reasons, Holmes [18] suggested planning the bunker starting from the daily feed-out rate, assuming that the next steps will ensure the total daily removal of the exposed face.

The importance of daily/weekly feed-out rate is made clear by the paper by Pitt and Muck [29]; they evidenced the benefit of a daily removal rate above 10.0 cm d^{-1} for the majority of the year, but they also recommended a removal rate of at least 15 cm d^{-1} during the warm season [29]. However, the same weekly removal rate could be attained by an alternate daily removal of 50% of the surface (or less), rather than a total daily removal (100% of the surface). The result is not the same, as confirmed by our study. In fact, the first strategy increases the average daily surface exposed to the air, allowing lateral air infiltration into the unremoved forage mass, at the limit of the other 50% of the removed surface. With the 100% daily surface removal, we accept (because it is almost unavoidable) the initiation of yeast activity in the first 15 cm (see the graphs by [29]), but removal within 24 h prevents the start of aerobic spoilage that can lead to a new rise in clostridia count. This seems consistent with our sampling scheme, where the lateral and upper spoiled silage was not included, and the mass core was the most represented share within the sample.

4.3. Silage Unloading Equipment

The best results being obtained in silos unloaded using a "front-end loader with cutter" may be explainable as a consequence of the most regular (smooth) surface being left after the daily silage removal. This outcome—well shown in Figure 5—is probably due to a different degree of mechanical action by the loader on the first layer of the silo face. It is common knowledge that a simple front-end loader acts mechanically on the mass

without a direct cut, therefore causing the movement of more than the removed volume. This way, the remaining silo face may become less compact in the first layer, enabling air penetration. During the Italian hot season, average daily temperatures above 27 °C from July to September 2018 promote the restart of clostridia activity. As only 31.3% of silos had a daily 100% face width removal in our study, we can argue that at least half of the remaining silo surfaces (50% daily removal) could be exposed to an average environmental temperature that is optimal for clostridia growth, for at least 48 h. Visser et al. [3] created their farm-level model considering a minimum growth temperature for butyric acid bacteria of 9 °C and an optimum at 37 °C; the consequence in the Italian summer climate may be easily arguable. The importance of a correct feed-out method was already reported by Heguy et al. [19] in their survey of California dairy producers; however, in their situation, there was a great share of farmers who removed silage from the face daily using a simple front-end loader—generally in systems where the pile (and not the bunker) is the most represented silo type.

The importance of silo linear feed-out speed (expressed as cm d^{-1} of advancement) was reported by Okatsu et al. [30] for corn silage within a pile system. In our survey, we did not observe a significant effect of the daily removal in depth, probably because of the limited number of pile silos (Table 2). It is likely that bunker silos (the majority in our study) are less prone to air penetration from lateral surfaces (since they have walls).

4.4. Bunker Silos

Even if the mean bunker silo length was within the maximum recommended value of 46 m [18], the high SD suggests that a large share of our samples had longer bunkers than recommended.

The age of the bunker silo (by age class) did not affect the mean value of spore-forming bacteria counts, even if an appreciable shift in their distribution toward higher values for the older silos was evident (Figure 6).

The substantial equivalence of the spore MPNld/g in the bunker silos between the three classes of age is explainable by attentive care for the integrity of the floor and, especially, of the lateral walls. This seems to be confirmed by the results from silos with different kinds of spoilage. In fact, considering our sampling procedure, it is reasonable to suppose that poorly sealed bunkers with lateral walls are more prone to lateral air infiltration during the storage period, likely because of partial subsidence during filling and subsequent fermentation in a poorly packed mass. Aerobic spoilage in the lateral mass of silos less than 8 years old might suggest worse compaction close to lateral walls, rather than inadequate plastic film management at the silo's closing.

A practical and quick system for classifying the kind of spoilage in the bunker silo could help the advisor and the farmer in the identification of the critical points in the silage-making process [21]. A higher spore content in bunker silos with lateral spoilage, as defined by the code "2" (see also, Figure 1a), suggests that a good maintenance of bunker wall integrity can help to prevent ANSB contamination in the summer period. It is important to remember that during summer months (namely July) we found that the final part of the corn silage mass had been harvested in the past year. Therefore, if the integrity of the lateral walls is damaged, air infiltration is possible for the course several months, and even during spring (which is sometimes very hot and humid in the Po Valley).

4.5. Farms

The main results from the analysis of the ANSB data suggest an important role of corn silage quality in the farm contamination chain of spore-forming bacteria. Surprisingly (see Table 4 for the regression analysis), fecal MPNld did not factor into the model, with TMR playing a minor role in the explanation of milk ANSB counts. A strong relationship was found between milk and corn silage spore counts.

This lack of a relationship between fecal and milk spore counts was compared with the results of Borreani et al. [14], who found positive correlations between these counts

only when spoiled silage was not (partially or fully) discarded, but not when it was. In our study, we did not assess the actual degree of spoiled silage removal. In addition to the varying extents of removal of the spoiled area from the silos, as in the quoted study by Borreani et al. [14], other factors such as bedding management and milking hygiene (not included in the present study) likely affected this result.

The significant (but moderate) relationship between the spore-forming bacteria counts in corn silage and those found in milk—confirmed by the regression analysis—only partially explain the complexity of the ANSB contamination chain at the farm level. The different silage contamination reflected the different extents of susceptibility to aerobic spoilage. The lack of a clear correlation between silage and TMR ANSB counts probably reflects the difficulty in accounting for the bias derived from the silage surface sampling. It is well known that the housing system, manure, and milking routine management are important points that may reduce the risk of milk contamination by these bacteria [3,4,9–11,14]; however, our results seem to confirm what is easily observable when employing the model proposed by Vissers et al. [3]: that it is very difficult to limit the on-farm spore-forming bacterial contamination chain if the feed (silage) spore count is very high.

Based on the results of the regression analysis, Figure 8 shows how the classification of farms according to the ANSB contamination of their corn silage confirms the lower risk of milk contamination when the corn silage ANSB count is below the threshold of 3.0 MPNld g^{-1}. This finding confirms the results of Vissers et al. [3], who reported an easy containment of spore-forming bacteria in the farms' milk tanks when silage contained less than 3 MPNld g^{-1}; they also underlined how the control measures in the contamination chain were ineffective when silage contained more than 5 MPNld g^{-1} of spores. When this critical point of silage quality is recognized, it should be more intuitive for the farmers to consider the possible economic return from a rational bunker silo dimensioning and its related investment. As reported by Holmes [18], a square silo requires the lowest initial investment compared to a narrow rectangle to store the same amount of forage, because the perimeter (the walls) increases in the latter. Our results suggest evaluating how the invested building cost for a longer bunker silo may be counterbalanced by the improved silage quality (in this case, microbiological) and the consequent gain in milk price attainment within the milk-quality-based payment system adopted by the Grana Padano PDO processors. A further motivation to improve bunker silo planning should be the reduction in dry matter losses during feed-out. In fact, an increased ratio between daily removed volume and exposed surface will ensure the reduction in DM losses, because this essentially takes place in the first 10 cm, within 24 h [29].

Ruppel et al. [31] found no differences in silage density related to particle size, suggesting that good packing activity may be unaffected by different possible choices in terms of the selected cutting action and length by the farmer (see the present concern about the increasing use of new cutting techniques, such as shredding); the only spoilage item negatively correlated ($p < 0.10$) with particle size in their study was the temperature of the upper silage, but not that of the silo face. Additionally, the negative relationship was strange, because it is generally assumed that silage porosity is a problem with long-cut forages, which are more difficult to pack; however, no correlation was found between particle size and aerobic instability.

5. Conclusions

The main results of our study were at the silo and farm levels. At the silo level, we showed how the distribution of silage mass by silo length, along with the total daily surface removal, may have an important joint effect in reducing contamination by spore-forming bacteria during the summer months in humid climates. Silo dimensions and their management also exert a partial (but significant) effect on the possibility of reducing contamination by spore-forming bacteria during the summer months in humid climates.

At the farm level, in TMR based on corn silage, the ANSB contamination of the silage is the most important factor within the silage–TMR–feces–milk contamination chain, affecting

the final contamination level of the product. We are not aware of other specific means of contamination by ANSB from silage to milk; however, since corn silage was the main quantitative source of ANSB at the beginning of this chain, this undoubtedly increased the potential contamination at the farm level.

Author Contributions: Conceptualization, F.A. and A.G.; methodology, F.A. and D.C.; formal analysis, F.A.; investigation, F.A., G.S., and F.P.; resources, F.A. and A.G.; data curation, R.M. and G.S.; writing—original draft preparation, F.A.; writing—review and editing, F.A., R.M., F.P., and D.C.; supervision, F.A.; project administration, F.A. and A.G.; funding acquisition, F.A. and A.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partially funded by Cremona Food-LAB project (Fondazione Cariplo, co-funded by Regione Lombardia), and by the AGRIDIGIT project, PLF4MILK subproject (D.M. 36507/7305/2018, Italian Ministry of Agricultural, Food, and Forestry Policies).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent from farmers was not necessary because the activity was conducted with the cooperation and under the supervision of the Consorzio Tutela Grana Padano within its institutional activities with associated members.

Data Availability Statement: Data were obtained in cooperation with the Consorzio Tutela Grana Padano, and are available from the authors with the permission of the third party.

Acknowledgments: We give special thanks to the Associazione Regionale Allevatori Lombardia (ARA Lombardia) technical staff for their support in farm survey management: Carlo Lovati, Paolo Marconi, and Lucio Zanini. We also give thanks to the Consorzio Tutela Grana Padano for its external support in the promotion and realization of this project. Furthermore, we thank Teresa Rutigliano and Andrea Bragaglio for their support in copying down and organizing the questionnaire data.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tabacco, E.; Comino, L.; Borreani, G. Production efficiency, costs and environmental impacts of conventional and dynamic forage systems for dairy farms in Italy. *Eur. J. Agron.* **2018**, *99*, 1–12. [CrossRef]
2. Borreani, G.; Coppa, M.; Revello-Chion, A.; Comino, L.; Giaccone, D.; Ferlay, A.; Tabacco, E. Effect of different feeding strategies in intensive dairy farming systems on milk fatty acid profiles, and implications on feeding costs in Italy. *J. Dairy Sci.* **2013**, *96*, 6840–6855. [CrossRef]
3. Vissers, M.M.M.; Driehuis, F.; Te Giffel, M.C.; De Jong, P.; Lankveld, J.M.G. Improving Farm Management by Modeling the Contamination of Farm Tank Milk with Butyric Acid Bacteria. *J. Dairy Sci.* **2006**, *89*, 850–858. [CrossRef]
4. Zucali, M.; Bava, L.; Colombini, S.; Brasca, M.; Decimo, M.; Morandi, S.; Tamburini, A.; Crovetto, G.M. Management practices and forage quality affecting the contamination of milk with anaerobic spore-forming bacteria. *J. Sci. Food Agric.* **2015**, *95*, 1294–1302. [CrossRef] [PubMed]
5. D'Incecco, P.; Faoro, F.; Silvetti, T.; Schrader, K.; Pellegrino, L. Mechanisms of Clostridium tyrobutyricum removal through natural creaming of milk: A microscopy study. *J. Dairy Sci.* **2015**, *98*, 5164–5172. [CrossRef] [PubMed]
6. Doyle, C.J.; Gleeson, D.; Jordan, K.; Beresford, T.P.; Ross, R.P.; Fitzgerald, G.F.; Cotter, P.D. Anaerobic sporeformers and their significance with respect to milk and dairy products. *Int. J. Food Microbiol.* **2015**, *197*, 77–87. [CrossRef] [PubMed]
7. Bermúdez, J.; González, M.J.; Olivera, J.A.; Burgueño, J.A.; Juliano, P.; Fox, E.M.; Reginensi, S.M. Seasonal occurrence and molecular diversity of clostridia species spores along cheesemaking streams of 5 commercial dairy plants. *J. Dairy Sci.* **2016**, *99*, 3358–3366. [CrossRef]
8. ISZ Istituto Zooprofilattico Sperimentale "Bruno Ubertini". Sporigeni Anaerobi. Available online: https://www.izsler.it/pls/izs_bs/v3_s2ew_consultazione.mostra_pagina?id_pagina=529 (accessed on 17 May 2020).
9. Miller, R.A.; Kent, D.J.; Boor, K.J.; Martin, N.H.; Wiedmann, M. Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. *J. Dairy Sci.* **2015**, *98*, 4338–4351. [CrossRef]
10. Bava, L.; Colombini, S.; Zucali, M.; Decimo, M.; Morandi, S.; Silvetti, T.; Brasca, M.; Tamburini, A.; Crovetto, G.M.; Sandrucci, A. Efficient milking hygiene reduces bacterial spore contamination in milk. *J. Dairy Res.* **2017**, *84*, 322–328. [CrossRef]
11. Evanowski, R.L.; Kent, D.J.; Wiedmann, M.; Martin, N.H. Milking time hygiene interventions on dairy farms reduce spore counts in raw milk. *J. Dairy Sci.* **2020**, *103*, 4088–4099. [CrossRef]
12. Calamari, L.; Morera, P.; Bani, P.; Minuti, A.; Basiricò, L.; Vitali, A.; Bernabucci, U. Effect of hot season on blood parameters, fecal fermentative parameters, and occurrence of Clostridium tyrobutyricum spores in feces of lactating dairy cows. *J. Dairy Sci.* **2018**, *101*, 4437–4447. [CrossRef]

13. Storm, I.M.L.D.; Kristensen, N.B.; Raun, B.M.L.; Smedsgaard, J.; Thrane, U. Dynamics in the microbiology of maize silage during whole-season storage. *J. Appl. Microbiol.* **2010**, *109*, 1017–1026. [CrossRef] [PubMed]
14. Borreani, G.; Ferrero, F.; Nucera, D.; Casale, M.; Piano, S.; Tabacco, E. Dairy farm management practices and the risk of contamination of tank milk from *Clostridium* spp. and *Paenibacillus* spp. spores in silage, total mixed ration, dairy cow feces, and raw milk. *J. Dairy Sci.* **2019**, *102*, 8273–8289. [CrossRef] [PubMed]
15. Vissers, M.M.M.; Driehuis, F.; Te Giffel, M.C.; De Jong, P.; Lankveld, J.M.G. Concentrations of Butyric Acid Bacteria Spores in Silage and Relationships with Aerobic Deterioration. *J. Dairy Sci.* **2007**, *90*, 928–936. [CrossRef]
16. Bernardes, T.F.; Daniel, J.L.P.; Adesogan, A.T.; McAllister, T.A.; Drouin, P.; Nussio, L.G.; Huhtanen, P.; Tremblay, G.F.; Bélanger, G.; Cai, Y. Silage review: Unique challenges of silages made in hot and cold regions. *J. Dairy Sci.* **2018**, *101*, 4001–4019. [CrossRef] [PubMed]
17. Ashbell, G.; Weinberg, Z.G.; Hen, Y.; Filya, I. The effects of temperature on the aerobic stability of wheat and corn silages. *J. Ind. Microbiol. Biotechnol.* **2002**, *28*, 261–263. [CrossRef] [PubMed]
18. Holmes, B.J. Software applications for sizing silos to maximize silage quality. In *Proceedings of International Symposium on Forage Quality and Conservation*; University of São Paulo: Piracicaba, Brazil, 2009; pp. 189–208.
19. Heguy, J.M.; Meyer, D.; Silva-del-Río, N. A survey of silage management practices on California dairies. *J. Dairy Sci.* **2016**, *99*, 1649–1654. [CrossRef]
20. McGilliard, M.L.; Crowgey, J.H.; Pecsok, S.R.; James, R.E.; Kohl, D.M. Comparisons of Costs After Tax of Storing Silages in Four Types of Structures. *J. Dairy Sci.* **1987**, *70*, 724–731. [CrossRef]
21. Mickan, F.J.; Martin, M.D.; Piltz, J.W. Silage Storage. In *Successful Silage*; Kaiser, A.G., Piltz, J.W., Burns, H.M., Griffiths, N.W., Eds.; Dairy Australia and New South Wales Department of Primary Industries: Orange, NSW, Australia, 2004; Chapter 9; pp. 217–252. ISBN 0734715835.
22. Gallo, A.; Bertuzzi, T.; Giuberti, G.; Moschini, M.; Bruschi, S.; Cerioli, C.; Masoero, F. New assessment based on the use of principal factor analysis to investigate corn silage quality from nutritional traits, fermentation end products and mycotoxins. *J. Sci. Food Agric.* **2016**, *96*, 437–448. [CrossRef]
23. Annibaldi, S. Modificazione della prova di Weinzirl per la ricerca dei clostridi butirrici nel latte. *Sci. Tec. Latt.* **1969**, *20*, 75–79.
24. Lodi, R.; Brasca, M.; Nardi, C.; Tamburini, A. Determinazione del contenuto di anaerobi sporigeni in latte e prodotti del settore lattiero-caseario [Detection of anaerobic spore formers in milk and dairy products]. *Il Latte* **1997**, *22*, 196–205.
25. Jarvis, B.; Wilrich, C.; Wilrich, P.-T. Reconsideration of the derivation of Most Probable Numbers, their standard deviations, confidence bounds and rarity values. *J. Appl. Microbiol.* **2010**, *109*, 1660–1667. [CrossRef]
26. R Core Team. R: A Language and Environment for Statistical Computing. Available online: http://www.sthda.com/english/wiki/correlation-matrix-an-r-function-to-do-all-you-need (accessed on 2 March 2020).
27. Mostafa, E.; Roesmann, M.; Maack, C.; Schmittmann, O.; Buescher, W. Automated pressure regulation for a silage bagging machine. *Comput. Electron. Agric.* **2020**, *173*, 105399. [CrossRef]
28. Borreani, G.; Tabacco, E. Low Permeability to Oxygen of a New Barrier Film Prevents Butyric Acid Bacteria Spore Formation in Farm Corn Silage. *J. Dairy Sci.* **2008**, *91*, 4272–4281. [CrossRef] [PubMed]
29. Pitt, R.E.; Muck, R.E. A Diffusion Model of Aerobic Deterioration at the Exposed Face of Bunker Silos. *J. Agric. Eng. Res.* **1993**, *55*, 11–26. [CrossRef]
30. Okatsu, Y.; Swanepoel, N.; Maga, E.A.; Robinson, P.H. Impacts of some factors that effect spoilage of silage at the periphery of the exposed face of corn silage piles. *Anim. Feed Sci. Technol.* **2019**, *247*, 234–247. [CrossRef]
31. Ruppel, K.A.; Pitt, R.E.; Chase, L.E.; Galton, D.M. Bunker Silo Management and Its Relationship to Forage Preservation on Dairy Farms. *J. Dairy Sci.* **1995**, *78*, 141–153. [CrossRef]