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The microbiota of ensiled forages and of bulk tank milk on dairy cattle farms in northern Sweden

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ABSTRACT

Factors contributing to variations in the quality and microbiota of ensiled forages and in bulk tank microbiota in milk from cows fed different forages were investigated. Nutritional quality, fermentation parameters and hygiene quality of forage samples and corresponding bulk tank milk samples collected in 3 periods from 18 commercial farms located in northern Sweden were compared. Principal coordinates analysis revealed that the microbiota in forage and bulk milk, analyzed using 16S rRNA gene-based amplicon sequencing, were significantly different. The genera *Lactobacillus*, *Weissella*, and *Leuconostoc* dominated in forage samples, whereas *Pseudomonas*, *Staphylococcus*, and *Streptococcus* dominated in bulk milk samples. Forage quality and forage-associated microbiota were affected by ensiling method and by use of silage additive. Forages stored in bunker and tower silos (confounded with use of additive) were associated with higher levels of acetic and lactic acid and *Lactobacillus*. Forage ensiled as bales (confounded with no use of additive) was associated with higher DM content, water-soluble carbohydrate content, pH, yeast count, and the genera *Weissella*, *Leuconostoc*, and *Enterococcus*. For bulk tank milk samples, milking system was identified as the major factor affecting the microbiota and type of forage preservation had little effect. Analysis of common amplicon sequence variants (ASV) suggested that forage was not the major source of *Lactobacillus* found in bulk tank milk.

Key words: forage quality, forage microbiota, ensiling method, bulk tank milk microbiota

INTRODUCTION

Microorganisms play important and beneficial roles in forage preservation and in processing of dairy products. The emergence of molecular methods for characterization of microbial community composition has opened up possibilities to identify and quantify the presence of microorganisms along the value chain of milk, from forage (McAllister et al., 2018) to the resulting dairy products (Decadt et al., 2023).

There is now increasing interest in evaluating the beneficial effects of the natural microbiome in forages and milk on the resulting dairy products (Manzocchi et al., 2021; Bettera et al., 2023). The intensification and modernization of dairy production, including the introduction of new technologies and changes to farm practices, has affected the intrinsic quality of milk and its microbial community composition. For example, variation in bulk tank milk microbiota between farms has been reported (Skeie et al., 2019) and a recent study by our research group showed clear effects of milking technology and farm routines on the microbial community in bulk tank milk (Sun et al., 2022). A recent study highlighted the effect of farm practices on milk quality and evaluated the possibility to adjust on-farm management to achieve raw milk with characteristics suitable for a specific end product of the value chain, such as cheese (Rey-Cadilhac et al., 2023).

The influence of feed, especially forages, on milk microbiota in the case of the Italian Parmigiano-Reggiano cheese has been reviewed by Mordenti et al. (2017). However, to our knowledge, few studies exist that linked variation in forage microbiota with that in bulk tank milk microbiota in silage-based feed systems in Scandinavia. Ensiling as a forage preservation method, often in combination with the use of additives (e.g., acids or microbial inoculants) has been common on-farm practice for many decades and is largely dependent on the anaerobic ac-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

Table 1. Farm data relating to milk production and type of forages fed to cows on the 18 participating farms; mean values for all farms and 3 sampling periods (P1, P2, P3); significance (*P*-value), SE, and number of observations (N) according to ANOVA general linear model

Variable	P1[1]	P2	P3	*P*	SE	N
Number of lactating cows[2] (herd size)	64	66	62	0.93	8.5	53
Milk production,[2] kg/cow per day	27.2	28.4	27.5	0.89	1.74	53
Milk fat content,[3] %	4.61	4.67	4.38	0.35	0.150	53
Milk protein content,[3] %	3.65	3.59	3.62	0.78	0.063	53
SCC,[3] 1,000/mL	168	162	211	0.14	18.7	53
Total bacteria,[3] 1,000/mL	7.0	11.3	11.1	0.30	2.15	53
Thermoresistant bacteria,[3] /mL	847	918	1,983	0.50	821.2	41
Forage from round bales,[4] %	53	57	70	0.58	11.9	51
Forage from tower silos,[4] %	24	22	12	0.70	9.9	51
Forage from bunker silos,[4] %	23	21	17	0.91	9.8	51
Forage in the diet,[4] % of total DMI	61	57	57	0.17	1.9	49

[1]Periods were as follows: P1 = November 2017; P2 = February and March 2018; P3 = September 2018.

[2]Data from animal recording (SNDHRS, Växa Sverige).

[3]Data from the official milk grading laboratory (Eurofins, Jönköping, Sweden).

[4]Data from interviews with the farmers.

tivities of lactic acid-producing bacteria (McDonald et al., 1991). Different agricultural practices have an effect on the microbiome in the ley crop, silage, cow housing environment and eventually also the milk (Gomes et al., 2020). Vacheyrou et al. (2011) found considerable contamination of milk by the stable environment, with milk having more microbial species in common with air in the farm environment and with teat surfaces than with hay samples. Gagnon et al. (2020) investigated the abundance of lactic acid bacteria (**LAB**) in raw milk in relation to the type of forage used to feed dairy cows on 24 farms by characterizing microbial communities in forage and bulk tank milk samples. The results revealed transfer of some strains from silage to milk, and forage was only a minor contributor of LAB to milk.

The aim of the present study was to characterize the microbiota present in forage and bulk tank milk on the selected farms, identify factors contributing to variations and evaluate to what extent the forage microbiota contributed to the microbiota in bulk tank milk on the farms.

MATERIALS AND METHODS

Farm and Animal Data

As a continuation of a previous larger survey by our research group, data from 42 commercial dairy farms in northern Sweden were collected and milk samples characterized (Priyashantha et al., 2021a,b; Sun et al., 2022), 18 of these 42 farms producing milk that varied in different aspects were selected for the present investigation. Forage and milk samples for analysis were collected on the selected 18 dairy farms in northern Sweden. The selection criteria and details of management regimen on these farms can be found in our previous publication (Sun et al., 2023). Eight of the farms had free stall and automatic milking system (**AMS**), 6 farms had a tiestall system (**TIE**), 3 farms had free stall and a milking parlor (**MP**) and one had a milking carousel (also considered as MP). The forage used was mainly grass silage, but also some whole crop cereals or cereals/peas. All farms except 2 participated in the Swedish national dairy herd recording scheme (**SNDHRS**), where data are gathered by the Swedish cattle farmers' association Växa Sverige. Milk data were collected once per month through a routine test milking.

All 18 selected farms were visited and sampled in 3 periods: November 2017, February/March 2018, and September 2018, hereafter called periods (**P1–P3**). Milk production data used for the study were acquired from SNDHRS if a test milking had been made within 10 d before or after each sampling period. If this was not the case, means of values from the closest test milkings before and after the period were used. Farm data used in the evaluation, including number of lactating cows and production parameters, are presented in Table 1. The percentage of different cow breeds in a herd was calculated from SNDHRS data. Holstein was the dominant breed, but cows of the Nordic Red, Swedish Mountain, and Swedish Jersey breeds were also used.

Sampling and Analyses of Milk

In Sweden, farm bulk milk is generally collected by the dairy plant every 2 d. Between each collection, the number of milkings was 3 to 4 for farms with tie-stalls or milking parlors. The temperature in the storage tanks averaged 4.2°C during collection. During each milk collection, a representative bulk milk sample is collected by a device installed in the truck. This sample is transported

refrigerated to a milk testing laboratory for analysis of milk quality parameters. For the purposes of this study, additional bulk milk samples (250 mL) were taken manually by the truck driver during ordinary milk collection on participating farms. This was done on 3 consecutive occasions (every second day) in each sampling period (i.e., in total 9 bulk milk samples per farm [3 periods × 3 sampling days]). The routinely collected milk samples were analyzed for total bacteria count (**TBC**) and thermoresistant bacteria count (**TRBC**) at Eurofins Steins Laboratory (Jönköping, Sweden). The TBC was determined using BactoScan FC (Foss) and TRBC was determined using a culturing method (Wehr et al., 2004). The corresponding manually collected tank milk samples were transported refrigerated to the Department of Molecular Sciences, Swedish University of Agricultural Sciences (**SLU**), Uppsala, for analysis of milk microbiota. Upon arrival, the samples were aliquoted into 2-mL tubes and stored as whole milk at −80°C until DNA extraction. The time from sampling on the farm to storage at −80°C was at most 30 h.

Sampling and Analyses of Forage

In the same week as the milk samplings were performed, the farms were visited, and the farmers were interviewed about the current situation regarding feedstuffs, rations, and so on. Grab samples around 2 kg of the forages fed to the cows were taken from several places in the bales or from the silo, using plastic gloves. In a few cases where this was not possible, samples were taken from the feed wagon in the barn. If the diet consisted of several different forages, samples were taken from each of the feed components. All forage samples were kept in plastic bags surrounded by ice pads during transportation to the laboratory (SLU, Umeå), where they were stored refrigerated. Samples from one farm at a time were taken out and placed in a clean plastic container, where they were mixed in the same proportions as used in the on-farm diet. Long stems were cut to facilitate mixing and correct subsampling. Three subsamples of weight around 600 g were saved in new plastic bags. One fresh subsample for hygiene analysis was transported using ice pads to SLU, Uppsala. Additional subsamples, one for analyses of nutritional values and fermentative qualities and one for microbiota analysis, were frozen at −18°C and transported under frozen conditions to SLU, Uppsala. The time from collecting the samples at the farm until they were placed in the freezer was at most 8 h. Upon arrival at SLU, Uppsala, the subsample for microbiota analysis was stored at −80°C until DNA extraction.

Hygiene analysis and analyses of nutritional and fermentative qualities were conducted at the Department of Animal Nutrition and Management, SLU, Uppsala. In the hygiene analysis, 30g of samples were transferred to a stomacher bag along with 270 mL of quarter-strength Ringer's solution with Tween 80 (O'Brien et al., 2007). The stomacher bag was processed for 120 s at normal speed in a stomacher (Seward, Worthing, UK). Then 10 mL aliquots of the resulting emulsion were used for serial dilutions and cultured on plates with different selective media and analyzed for yeast (malt extract agar) *Clostridia* spores (reinforced clostridial agar), mold (malt extract agar), *Enterobacteria* (violet red bile dextrose) and LAB (Rogosa agar, where mainly *Lactobacillus* grow but also *Pediococcus* and *Leuconostoc*, and Slanetz-Bartley (**S-B**) agar, which mainly shows growth of *Enterococcus*). For LAB, the lowest detectable number was either 2.7 (Rogosa) or 3.7 (S-B) log cfu/g sample. For the other microorganisms, the lowest detectable amount was 1.7 log cfu/g sample. For analytical results stated as <3.7, a value of 3.5 was used in calculations to facilitate computation of results (values <1.7 and <2.7 were handled similarly). All growth media were purchased from Merck Life Science AB (Solna, Sweden).

The first task in analysis of nutritional and fermentation qualities of forage was DM determination. For this, coarsely ground samples of fresh matter and conserved forages (nominal length 4 cm) were dried for 18 h at 60°C. After air equilibration, the samples were weighed and ground on a hammer mill to pass a 1.0-mm sieve and then dried again for 20 h at 103°C. Ash content was determined by incinerating the dried samples in an oven at 550°C for 3 h and then cooling in a desiccator before weighing. Concentration of total water-soluble carbohydrates (**WSC**) was determined as previously described (Larsson and Bengtsson, 1983). Total nitrogen (N) was determined according to the Kjeldahl method (Nordic Committee on Food Analysis, 1976), using a 2020 Digester and a 2400 Kjeltec Analyzer (FOSS Analytical A/S, Hilleröd, Denmark), and CP was calculated as N × 6.25. Neutral detergent fiber was analyzed as previously described (Chai and Udén, 1998).Acetic, lactic, propionic, and formic acid, ethanol, 2,3-butanediol, and ammonia-N in forage samples were analyzed in juice extracted from the forage in a hydraulic press. The samples were mixed 1:1 (wt/wt) with distilled water, frozen for 24 h, and then thawed before juice extraction. The concentrations of acetic, lactic, propionic and formic acid, ethanol, and 2,3-butanediol were determined by HPLC as previously described (Andersson and Hedlund, 1983). The pH of the diluted juice was measured with a pH meter (Metrohm 654, Metrohm, Herisau, Switzerland) fitted with a glass electrode. Ammonia-N concentration was determined using flow injection analysis (Tecator FIA 5010, Tecator, Höganäs, Sweden; Ruzicka and Hansen, 1988).

DNA Extraction from Forage and Milk

To prepare the materials for DNA extraction, the frozen forage samples were thawed at 4°C overnight and cut into pieces ~1 cm in length using sterilized scissors. The forage was then mixed and 30 g of the mixed sample were weighed into a stomacher bag. The stomacher bag was filled with 270 g of quarter-strength Ringer's solution with Tween 80 (O'Brien et al., 2007) and processed using a stomacher (Seward) at normal speed for 120 s. An aliquot (12 mL) of the homogenized slurry obtained was transferred to a 15-mL Falcon tube and centrifuged at 10,000 × *g* for 10 min at 4°C. The slurry pellet was then transferred to a 2-mL cryo-tube by adding 1 mL fresh quarter-strength Ringer's solution with Tween 80. The cryo-tube was centrifuged at 13,000 × *g* for 5 min at 4°C. The supernatant was discarded and the slurry pellet in the cryo-tube was stored at −80°C until use. Each forage sample was prepared for duplicate DNA extractions. Before the DNA extraction, the frozen samples in cryo-tubes were thawed at room temperature for 30 min. The DNA extractions were conducted with a NucleoSpin Soil DNA kit (Macherey-Nagel, Dueren, Germany) following the manufacturer's protocol, using a FastPrep-24 instrument (MP Biomedicals, Irvine, CA) at a speed of 5 m/s for 30 s, with lysis conditions adjusted with 700 µL SL2 + 150 µL SX Enhancer provided with the kit. Buffer SE (70 µL) provided with the extraction kit was used for DNA elution. Due to practical problems, the following forage samples were missing: farm 3, period 1; farm 8, period 3; and farm 17, period 3.

The DNA in milk samples was extracted as previously described (Sun et al., 2023). In brief, milk samples were thawed at 37°C for 15 min in a heating block. The cell pellets and fat were collected after centrifuge of thawed milk samples at 13,000 × *g* for 15 min at 4°C. DNA extraction was conducted using PowerFood DNA isolation kit (Qiagen AB, Sollentuna, Sweden) from the resulting cell pellets and fat following manufacturer's instructions.

Amplicon Library Preparation, Sequencing, and Bioinformatic Analysis

A 16S rRNA sequencing library was constructed with primers 515F and 805R targeting V4 region (Hugerth et al., 2014). Negative PCR controls and negative DNA extraction controls were both included in the sequencing library. Illumina adaptors and barcode were used for amplification, following a 2-step PCR approach described previously (Sun et al., 2019). The 16S rRNA library was sequenced using the Illumina Miseq platform at SciLifeLab (Stockholm, Sweden). The raw sequencing data have been deposited at the National Center for Biotechnology Information database, under accession number PRJNA1010645. Bioinformatic data processing was performed using Quantitative Insights into Microbial Ecology 2 (Core 2020.11; Bolyen et al., 2019). The raw demultiplexed reads were trimmed using Cutadapt to remove primer sequences (Martin, 2011). The trimmed reads were further processed using DADA2 to de-noise and de-replicate reads, merge pair end reads, and remove chimeras (Callahan et al., 2016), using a truncation length of 210 and 160 bp for forward and reverse reads, respectively. A phylogenetic tree was built using FastTree and MAFFT alignment (Katoh et al., 2002; Price et al., 2010). The SILVA SSU Ref NR 99 138 data set was first trimmed to the corresponding primer region and trained as classify-sklearn taxonomy classifier (Pedregosa et al., 2011; Quast et al., 2013; Bokulich et al., 2018). Amplicon sequence variants (**ASV**) were assigned taxonomy using the resulting classifier. The sequence analysis generated on average 75,383 (range 27,031–184,635) sequences from the forage microbiota data set and 22086 (range 4,238–99,637) sequences from the bulk milk data set. The ASV table was rarefied at 27,031 and 4,238 reads per sample for forage and bulk milk samples, respectively.

Statistical Analysis

Farm data, including SNDHRS and forage data, were analyzed using the statistical software NCSS (version 9, NCSS Statistical Software). For sequencing data, Kruskal-Wallis rank test followed by Dunn's test for pairwise comparisons with Benjamini and Hochberg correction was used to identify statistically significant differences in the observed ASV and in Shannon index values associated with farm factors. Principal coordinate analysis (**PCoA**) was used to study clustering patterns in forage microbiota and bulk milk microbiota, to identify farm factors of importance for microbiota composition in forage and bulk milk. The PCoA was based on Bray-Curtis metrics generated from ASV data, where ASV represented by fewer than 5 sequences in the data set were excluded. Analysis of similarity (ANOSIM) at 9,999 permutations was used for validation of clustering of forage microbiota caused by forage management factors. The following factors were evaluated: type of forage (grass silage, grass and whole crop silage, grass silage and pasture), method of preservation (bales, bunker silo, tower silo) and use of additives (yes, no). To evaluate associations between the microbiota (based on amplicon sequencing) and chemical and microbiological (culture based) parameters in the forage samples, principal component analysis (**PCA**) was used. In PCA, microbiota data at genus level were used and only genera represented by relative abundance higher than 0.1% in the data set were analyzed, using the software Simca (Version 18.0.0.372, Umetrics, Sartorius). Orthogonal projections to latent structures

discriminant analysis (**OPLS-DA**) and cross-validation were used to evaluate the effect of additives with Simca. Genera with CI >0 and predictive variable importance for the projection >1 were used for model interpretation (Galindo-Prieto, 2017). Number of observations (n), number of variables (K), and predictive performance (Q^2Y) are reported for the OPLS-DA model. To evaluate differences in TBC and TRBC between type of milking system, a mixed effects linear model was created in R version 4.2.3 (R Core Team, 2021), using milking system as fixed effect and farm as random effect.

Matched analysis was used to identify associations between forage microbiota and bulk tank milk microbiota. First, a search was made for matched pairs of forage and bulk tank milk samples (i.e., samples collected from the same farm and in the same sampling period). In total, 50 matched pairs were identified and included in the analysis. Next, ASV that were unique either to bulk tank milk or forage samples were filtered out from the ASV data. The remaining ASV were converted to binary data and ranked based on number of occurrences in both sample types.

RESULTS

Forage Preservation on Participating Farms

Of the forages fed to dairy cows on the 18 participating farms in the 3 sampling periods, on average 86% was grass silage, whereas whole crop silage (wheat, barley or mixtures of cereals and peas) constituted on average 10%. On one farm, hay was used in varying proportions. On most visits, it was found that more than one forage was used at the same time in the diet (e.g., first and second harvest bales or grass silage plus whole-crop silage). At most, 4 different forages were fed at the same time. We observed no significant difference between the 3 sampling periods in percentage of forage from different harvest occasions or percentage of grass or whole-crop silage.

On 15 of the 18 farms investigated, all or part of the forage was preserved as bales. Two farms used only tower silos and one farm only bunker silos for their silage. However, several farms stored their forage both as silage in bunker or tower silos and as bales, and the different silages were fed in varying proportions. Of the farms feeding bales, none used an additive at ensiling, whereas additives were always used when ensiling in bunker and tower silos. Acid additives were used on 7 farms, whereas 1 farm used a salt-based additive. No microbial additive was used in any of the farms investigated, nor was any combination of different additive types used. One farm (Farm 4) used silage stored in a bunker silo in periods 1 and 2, but switched to bales during period 3. Farms 11 and 12 used silages stored in tower silos in periods 1 and 2, but switched to bales during period 3.

The average percentage of forage in the diet was estimated for each farm in each period and varied between 45 and 77% of total DM intake, with no significant difference between sampling periods. In the third period (September 2018), the cows were still on pasture on 13 of the 18 farms. On 8 of those, the cows were out mainly for exercise and were fed at least 80% of their normal indoor ration. On the remaining farms with grazing cows, the indoor diet contributed 0–50% of the normal indoor ration.

Hygiene analysis and analyses of fermentation and nutritional qualities generally did not indicate inferior forage quality and no result was outside acceptable limits (Table 2). However, several significant differences in forage quality parameters were observed between sampling periods and between preservation methods (Table 2). Further evaluation of the results revealed that ensiling method and use of an additive were clearly confounded factors (i.e., ensiling the crop as bales involved no use of additive, whereas ensiling in tower and bunker silos always involved use of a silage additive). As expected, DM content was higher in bale silage, because biomass for bales is normally dried for longer in the field before storing. The lower DM in silages preserved in tower and bunker silos was also linked to more extensive fermentation. Acetic and lactic acid content were thus higher in the silages stored in bunker and tower silos, whereas bale silage had a higher content of WSC, pH, ethanol, yeast count and enterococci count (on S-B medium).

Overview of Forage and Milk Microbiota

Differences between forage and bulk milk microbiota were evaluated using Bray-Curtis distance comparison. The results revealed that the microbiota present in farm bulk milk was significantly different ($P < 0.001$) from that in the forage samples (Figure 1a). Alpha diversity measured as number of observed ASV (Figure 1b), and Shannon index (Figure 1c) were both significantly higher in bulk tank milk than in forage samples ($P < 0.001$ in both cases). Microbiota composition in the forage samples was dominated by the genera *Lactobacillus*, *Weissella*, and *Leuconostoc*, whereas the bulk tank milk microbiota was dominated by the genera *Pseudomonas*, *Staphylococcus*, and *Streptococcus* (Figure 2a).

Forage Microbiota and Farm Factors of Importance

PCoA using Bray-Curtis matrix showed no clear clustering pattern by farm or by sampling period for forage microbiota (Figure 3a). However, comparison of the 3

Table 2. Mean value of nutritional quality and fermentation parameters and hygiene quality of forages sampled on participating farms, presented by sampling period (P1–P3) and method of forage preservation; significance (*P*-value), SE, and number of observations (N) according to ANOVA general linear model[1]

	Period[2]					Forage preservation				
Variable[3]	P1 (n = 17)	P2 (n = 17)	P3 (n = 15)	*P*[4]	SE	Bales (n = 30)	Bunker silo (n = 9)	Tower silo (n = 10)	*P*[4]	SE
DM, %	36.2	37.9	44.2	0.082	2.52	41.8^{a}	31.2^{b}	38.9^{ab}	0.026	2.74
CP, g/kg DM	126	136	127	0.367	5.8	122^{b}	146^{a}	137^{ab}	0.011	6.0
NDF, g/kg DM	521	517	511	0.788	10.8	519	511	513	0.850	12.1
WSC, g/kg DM	56.2^{ab}	42.3^{b}	94.9^{a}	0.017	12.68	85.9^{a}	22.8^{b}	31.5^{b}	0.000	13.16
Ash, g/kg DM	66.8	70.5	66.8	0.553	2.77	66.9	69.8	70.0	0.653	3.11
pH	4.22^{b}	4.25^{b}	4.76^{a}	0.006	0.126	4.67a	3.89^{b}	4.01^{b}	0.000	0.122
Acetic acid, % of DM	1.24^{a}	1.21^{a}	0.66^{b}	0.041	0.171	0.68^{b}	1.79^{a}	1.51^{a}	0.000	0.153
Lactic acid, % of DM	4.16^{ab}	5.09^{a}	2.47^{b}	0.018	0.617	2.60^{b}	7.18^{a}	5.16^{a}	0.000	0.558
Propionic acid, % of DM	0.14^{a}	0.08^{b}	0.12^{ab}	0.021	0.015	0.10	0.13	0.13	0.324	0.018
Formic acid, % of DM	0.26^{a}	0.12^{ab}	0.05^{b}	0.020	0.053	0.03^{b}	0.32^{a}	0.33^{a}	0.000	0.050
Ethanol, % of DM	0.55	0.52	0.41	0.287	0.061	0.56^{a}	0.46^{ab}	0.33^{b}	0.023	0.064
2,3 Butanediol, % of DM	0.14	0.17	0.10	0.218	0.029	0.16	0.13	0.09	0.342	0.033
Ammonia-N, % of total N	4.45^{a}	4.84^{a}	2.56^{b}	0.002	0.453	3.46	5.09	4.66	0.055	0.544
Mold, log cfu/g fresh sample	2.13	1.89	2.10	0.652	0.199	1.71^{b}	2.12^{b}	2.95^{a}	0.000	0.177
Yeast, log cfu/g fresh sample	4.08^{ab}	2.98^{b}	4.53^{a}	0.008	0.346	4.43^{a}	3.09^{b}	2.73^{b}	0.001	0.372
Enterobacteria, log cfu/g fresh sample	1.63^{b}	1.50^{b}	3.15^{a}	0.000	0.246	2.32	1.57	1.66	0.140	0.331
Clostridia, log cfu/g fresh sample	1.87	1.65	1.98	0.302	0.150	1.71	2.20	1.86	0.104	0.164
LA_rog, log cfu/g fresh sample	7.81	6.92	6.91	0.057	0.300	7.36	7.06	6.96	0.634	0.354
LA_sb, log cfu/g fresh sample	5.19^{ab}	4.46^{b}	5.95^{a}	0.046	0.405	5.90^{a}	3.94^{b}	4.10^{b}	0.000	0.407

^{a,b}Values within rows with different superscripts differ significantly between periods/forage preservation methods.

[1]WSC = water-soluble carbohydrates; LA_rog = bacteria cultured on Rogosa agar; LA_sb = bacteria cultured on Slanetz-Bartley (S-B) agar.

[2]Periods were as follows: P1 = November 2017; P2 = February and March 2018; P3 = September 2018.

[3]Samples from Farm 18 in P2 and P3 removed due to a mixture of silage from bunker silo and bale; samples from Farm 8 in P3 removed due to using only hay as forage; and samples from Farm 17 in P3 removed due to providing no forage other than pasture to the cows.

[4]$P < 0.05$ considered to indicate a significant difference.

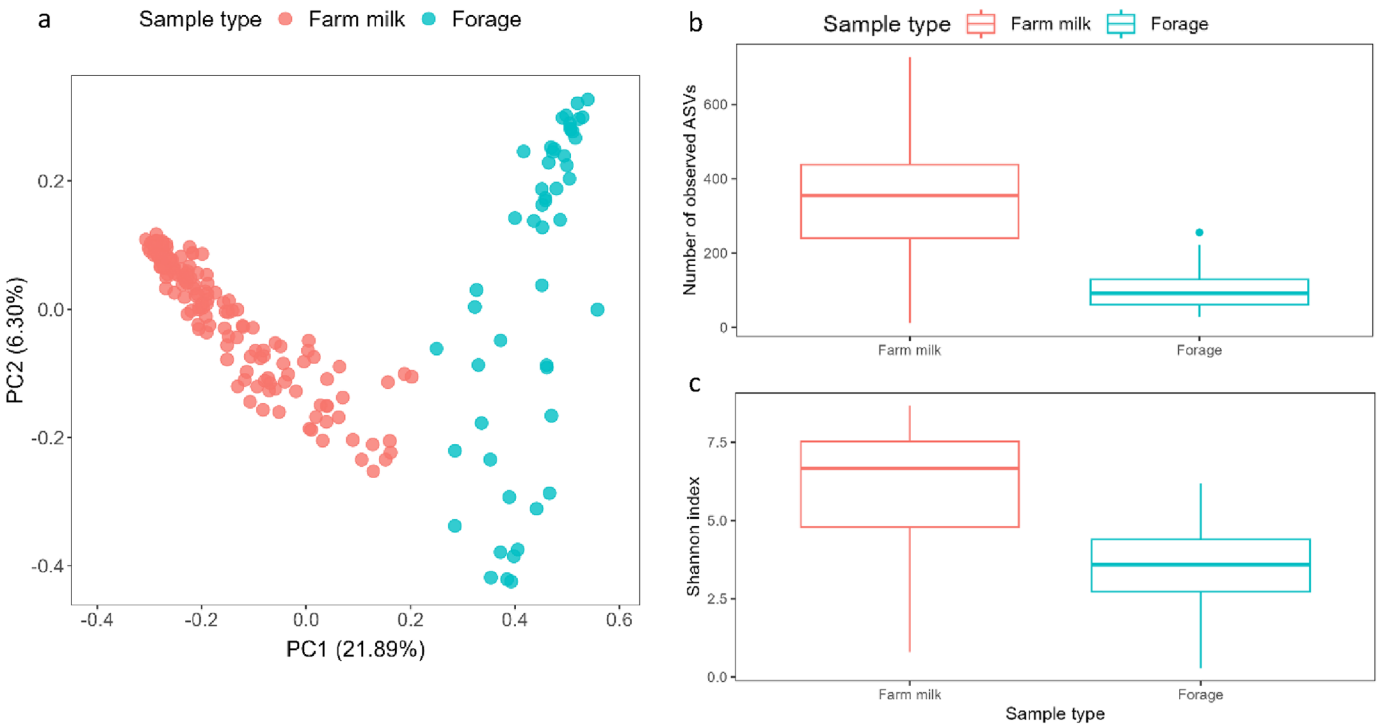

Figure 1. (a) Principal coordinate analysis (PCoA) plot of the microbiota in forage samples (n = 105) from 18 participating farms and the corresponding bulk tank milk samples (n = 165) collected during 3 periods, evaluated using Bray-Curtis distance matrix, colored by sample type ($P = 0.0001$), (b) boxplot of the number of observed ASV in forage and bulk tank milk samples ($P < 0.001$) and (c) boxplot of Shannon index in forage and bulk tank milk samples ($P < 0.001$). The horizontal line in each box is the median value, and the lower and upper edges of the boxes indicate the 25th and 75th percentiles of the distribution, respectively. Whiskers indicate the maximum and minimum values, dots represent values considered as outliers.

forage samples obtained from the same farm revealed higher similarity between periods 1 and 2 for several farms, whereas period 3 was located further away in the PCoA plot (Figure 3a), indicating that samples from period 3 had a deviating microbiota composition. Of the farm factors evaluated, those with the strongest effect on microbial community composition were silage preservation method and use of additives (Figure 3b). Regarding preservation method, forage samples from bales clustered separately from those from tower and bunker silos ($P < 0.001$), although we observed no difference in microbiota composition between samples stored in bunker and tower silos ($P > 0.05$). We observed a clear separation ($P < 0.001$) in the microbiota profiles between samples with and without use of additives (Figure 3b). However, this separation could not be distinguished from the effect of preservation method, because additives were never used in bales but always in silages stored in tower or bunker silos. Preservation method and use of additives also had an effect ($P < 0.01$) on alpha diversity measured by Shannon index (Supplemental Figure S1a, see Notes), but not on number of observed ASV. Bale silage (i.e., silage without additive, had a higher Shannon index; Supplemental Figures S1a and S1b) than bunker or tower silage.

Associations Between Microbiota Composition and Conventional Forage Evaluation Parameters

In agreement with the PCoA ordination of the 16S microbiota data, the PCA model that included nutritional and fermentation variables as well as 16S sequence and culture-based microbial parameters showed clear separation between samples collected from bales and samples collected from either bunker or tower silos (Supplemental Figure S2, see Notes). From the loading plot (Figure 4), it was evident that samples from bunker and tower silos were associated with high relative abundance of *Lactobacillus*, high content of organic acids (mainly lactic and acetic acid) and higher content of ammonia-N. In contrast, samples from bales were associated with higher DM, pH, and content of yeast and WSC. Moreover, the microbiota in bale samples clearly differed from that in bunker and tower silo samples and was negatively associated with the genus *Lactobacillus*, but positively correlated with several other bacterial taxa, including *Leuconostoc* and *Enterococcus* (Figure 4). In agreement with the PCA data, the OPLS-DA model (n = 49, K = 42, $Q^2Y = 0.666$, $P < 0.001$) showed that *Lactobacillus* genus was present in higher relative abundance in forage

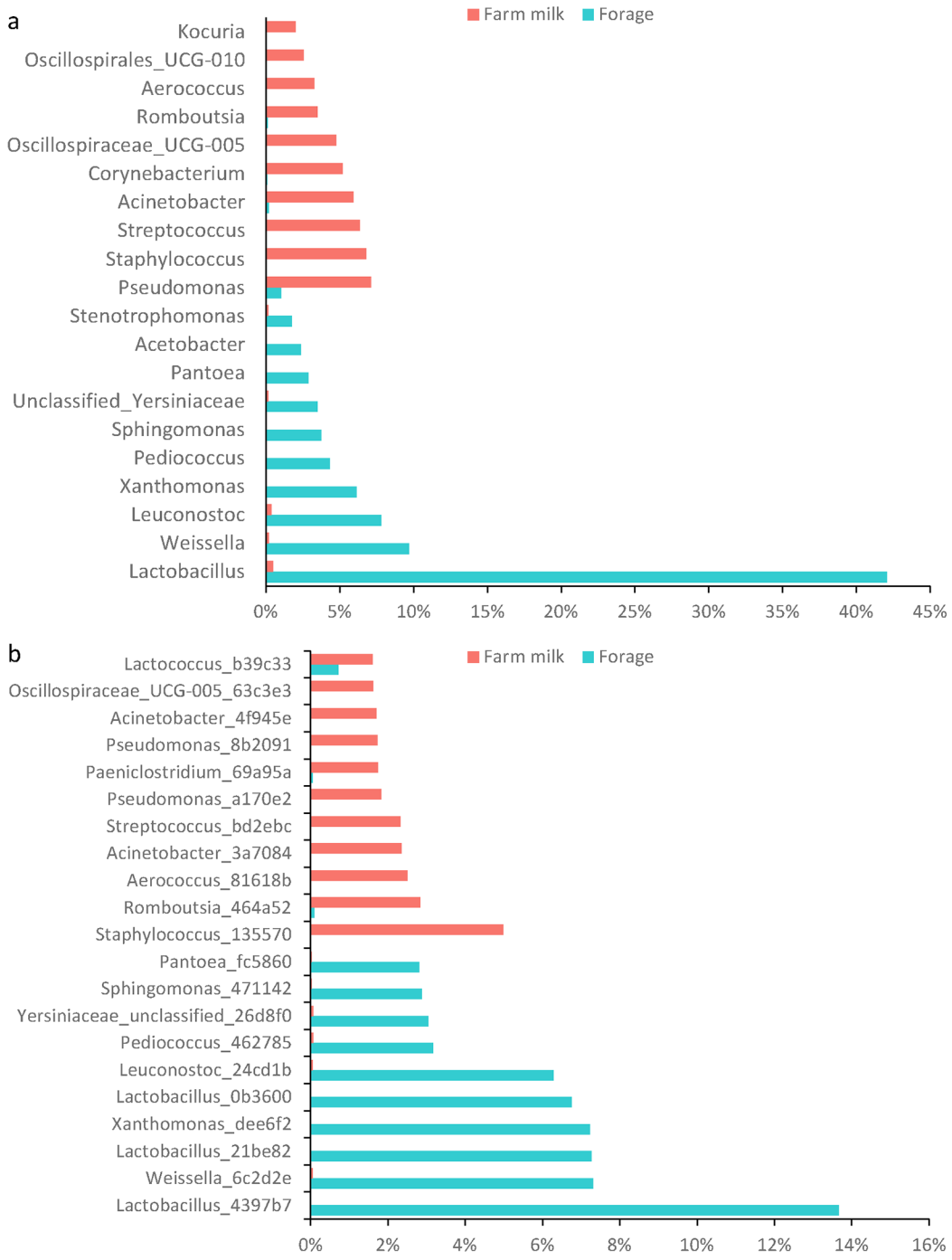

Figure 2. Relative abundance (%) distribution of the 10 most abundant (a) genera and (b) amplicon sequence variants in forage samples (n = 105) and in the corresponding farm bulk tank milk (n = 165).

treated with additive (confounding with bunker and tower silo; Supplemental Figure S3, see Notes), whereas *Weissella*, *Leuconostoc*, unclassified *Yersiniaceae*, *Hafnia-Obesumbacterium* group, and *Lactococcus* were present in higher relative abundance in forage samples without additive treatment (confounding with bale; Figure 5).

Bulk Tank Milk Microbiota

To assess whether factors identified as important for forage microbiota composition also influenced bulk tank milk microbiota, the potential effect of forage type on-farm (i.e., forage ensiled in bales, bunkers, or tower silos)

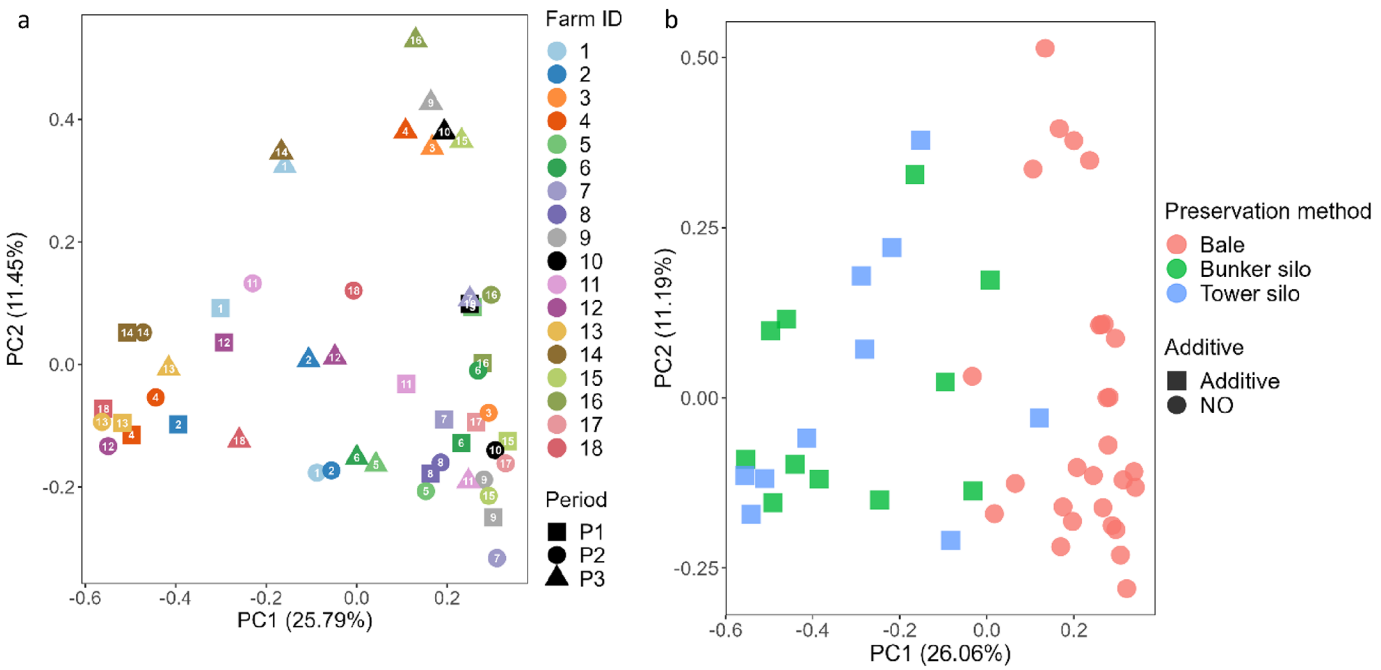

Figure 3. Principal coordinate (PC) analysis plot evaluated using Bray-Curtis distance matrix of microbiota of silage samples from 18 farms collected on 3 different occasions (periods 1–3), (a) colored by farm ID, shaped by period and (b) colored by forage preservation method ($P = 0.0001$, bunker vs. tower; not significant, bale vs. bunker; $P = 0.00015$, bale vs. tower; $P = 0.00015$), shaped by additive (additive, $P = 0.0001$). Farm 3 data removed from (b) due to missing additive information.

on bulk tank milk microbiota composition was evaluated (Figure 6a). This analysis revealed a more overlapping pattern, but with a difference in bulk milk microbiota between farms using silages stored in bunkers and farms using silages stored in tower silos ($P < 0.001$).

Evaluation of the effect of milking system on bulk tank milk microbiota revealed clear separation linked to milking system ($P < 0.001$), with the microbiota in bulk milk differing between farms using TIE stall milking, MP and AMS (all $P < 0.05$; Figure 6b). Moreover, total bacterial count data revealed that milk from AMS farms had higher TBC (log 4.01 ± 0.30 /mL) than milk from MP and TIE farms (log 3.69 ± 0.17 /mL and log 3.73 ± 0.26 /mL, respectively; $P < 0.05$).

Associations Between Forage and Bulk Tank Milk Microbiota

We observed a clear difference in microbial community composition between the 2 sample matrices (i.e., forage and bulk tank milk). The ASV present in highest relative abundance in forage samples were not the same as in bulk tank milk, and little overlap was present (Figure 2b). The dominant ASV in forage microbiota were *Lactobacillus*, *Weissella*, *Leuconostoc*, and *Xanthomonas* (Figure 2b), and these were found in very low relative abundance in the bulk tank milk samples. The bulk tank milk samples were instead dominated by ASV belonging to *Staphylococcus*, *Romboutsia*, *Aerococcus*, *Acinetobacter* and *Streptococcus* (Figure 2b), but these ASV were present in very low relative abundance in the forage samples.

To assess potential transfer of bacteria from forage to bulk tank milk, the extent to which ASV were present in both sample matrices was analyzed. This analysis, which was carried out on 50 forage and bulk tank milk sample pairs, did not take relative abundance into consideration (Table 3). *Lactococcus* b39c33 (43/50), *Leuconostoc* 24cd1b (29/50) and *Romboutsia* 464a52 (27/50) emerged as the ASV with the highest prevalence in the sample pairs, but no ASV was present in all sample pairs. Taking relative abundance into consideration, it was evident that the abundance of ASV common to both forage and bulk milk samples differed considerably between the sample types, except for *Lactococcus* b39c33 (Figure 2b).

DISCUSSION

The quality of the forages used by participating farms was generally acceptable in hygiene, nutritional, and fermentative quality. However, differences in forage nutritional quality and microbiota were observed between sampling periods and ensiling methods. One explanation for the observed differences is that on several farms, forages used in periods 1 and 2 originated from the same cut

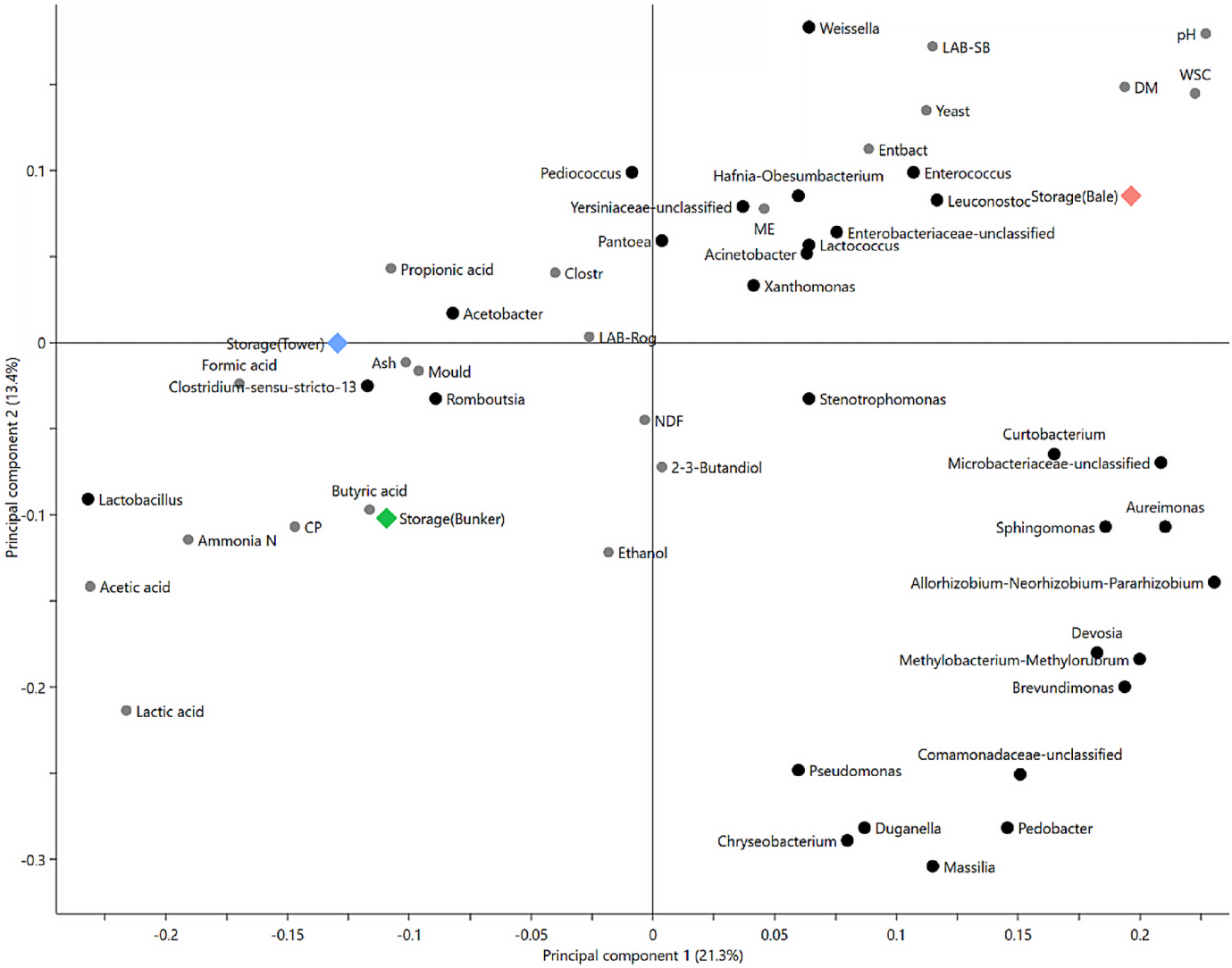

Figure 4. Loading plot from principal component analysis including the top 30 genera in forage (black circle) and nutritional, fermentation and hygiene quality parameters (gray circle). The 3 different methods used for forage preservation (diamonds) are indicated in different colors. Ammonia N = ammonia-nitrogen; WSC = water-soluble carbohydrates; LAB-Rog = bacteria cultured on Rogosa agar; LAB-SB = bacteria cultured on Slanetz-Bartley agar; Clostr = *Clostridia* spores cultured on reinforced clostridial agar; Entbact = bacteria cultured on violet red bile dextrose agar.

and type of preservation method. In addition, the biomass sampled in period 3 was harvested after an extremely warm and dry summer in Sweden (Eliasson et al., 2023), which probably had an effect. In an Irish study by Ferris et al. (2022), 68% of the farmers identified adverse weather as having a large or very large effect on the quality of their silage, whereas only 9% of farmers regarded the effect of weather-related delays on grass cutting as minor. In a Norwegian study, Randby and Bakken (2021) compared the quality of grass silage obtained from bales and from bunker silos, with or without application of an acid-based silage additive, and found that bale silages were more restrictedly fermented than bunker silages, resulting in higher concentrations of WSC and lower concentrations of ammonia-N. Previous studies have demonstrated the effectiveness of acid application in facilitating a rapid decline in pH, favoring the growth of epiphytic LAB, but with higher rates of acid application restricting the extent of fermentation, thereby increasing the concentration of residual WSC (Bernardes et al., 2018). The silages samples from bunker and tower silos in the present study, which were always produced with an additive, had lower WSC content than expected, but the rate of application of acid or salt additive is not known.

The 16S rRNA gene sequencing approach has been increasingly used and accepted in the past decade, but few previous studies have used it to compare the microbiota present in milk and in forage fed to the cows.

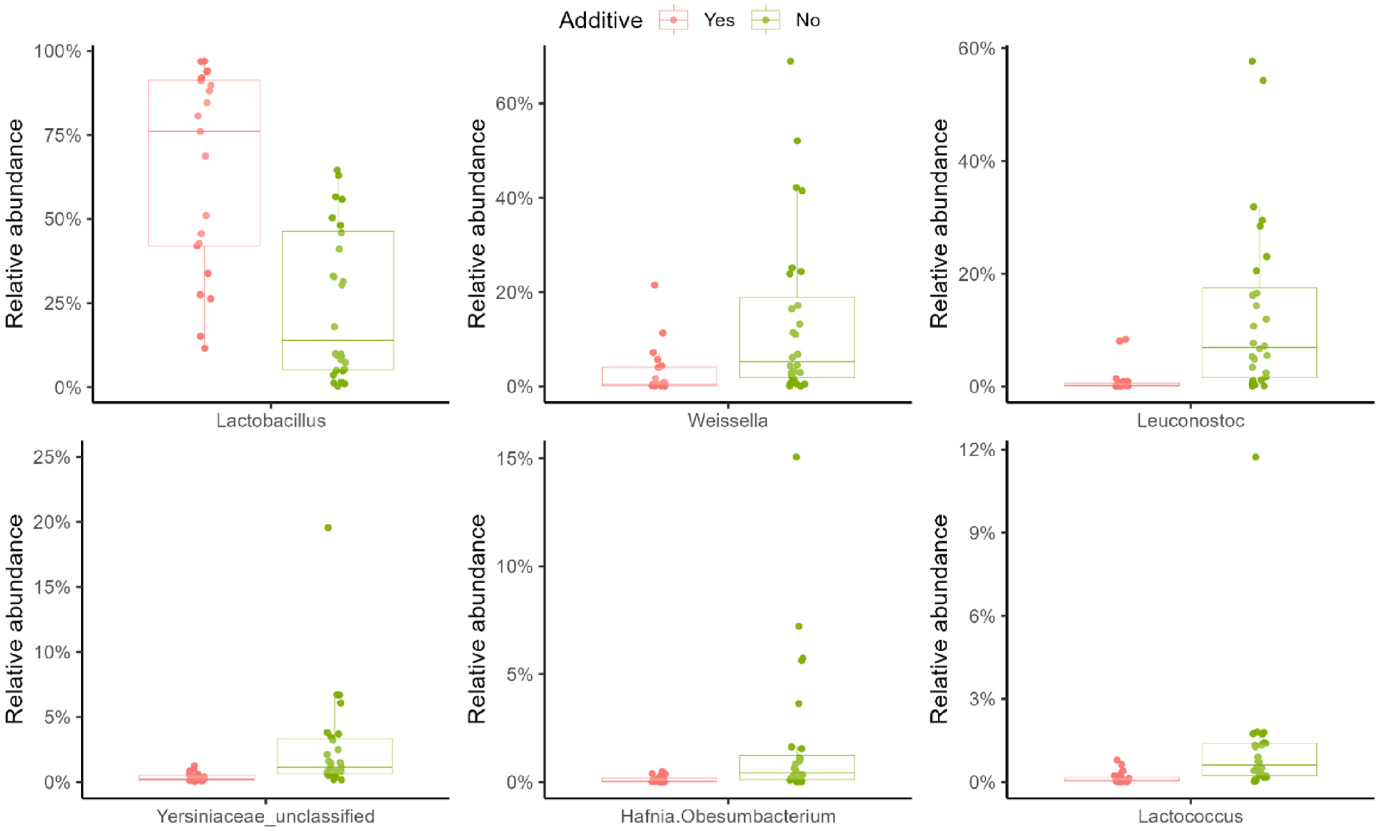

Figure 5. Relative abundance (%) of genera present in forage treated with (Yes) and without (No) additives, as identified in orthogonal projections to latent structures discriminant analysis (OPLS-DA; R2 = 0.666, Q2 = 0.468), with predictive variable importance for the projection >1. The lower and upper edges of boxes indicate the 25th and 75th percentiles of the values and the line in each box is the median (50th percentile). Dots represent data points of the relative abundance.

In this study, the microbiota present in bulk tank milk was very different from that in forage (Figure 1a) and alpha diversity, measured as number of observed ASV, and Shannon index were higher in milk than in forage. In contrast, a study by Gomes et al. (2020) using phylogenetic species variability (**PSV**) as a measure of alpha diversity observed higher PSV in silage compared with milk. In that study, the flow of microorganisms across milk production stages was investigated by sequencing microbial communities in soil, silage, manure, and milk samples from organic and conventional dairy farms. The results showed no clear effect of agricultural system on the microbiota in silage and milk from the farms (Gomes et al., 2020). A study by Doyle et al. (2017) observed only minor contributions from grass or silage to the raw milk microbiota, but found that microorganisms on teat surfaces and in feces were major sources. In the study by Ouamba et al. (2023), 113 ASV were found to be shared between forage rations and corresponding raw milk, yet the bacteria from forage represented a low proportion of the associated raw milk microbiota. Additionally, the authors found that silage-based forage rations share more ASV with raw milk, compared with a hay-based ration. In a recent survey (Sun et al., 2022), we found that milking system and teat preparation routine before milking had a strong effect on the microbiota in bulk tank milk. In the present study, milking system had a major effect on microbiota composition and on TBC level.

To identify factors contributing to observed differences in forage microbiota, forage production and the corresponding forage data were evaluated. As mentioned, forage preservation method and use of a silage additive were identified as the most important factors for forage microbiota composition. However, these 2 factors were confounded in the analysis and their individual effects were impossible to distinguish. Forage preserved as bales was associated with higher DM, pH, and WSC (Figure 4), explained by use of drier herbage for bales and by the plastic wrap applied at harvest creating a more airtight environment than for silage stored in tower and bunker silos (Spörndly and Nylund, 2017). The higher pH and WSC indicated that less fermentation occurred when forage was preserved as bales, whereas silages produced and stored in bunker and tower silos showed a higher degree

Table 3. Prevalence of amplicon sequence variants (ASV) in 50 pairs of tank milk and forage samples from the same farm and period

Amplicon sequence variant	Number of pairs
*Lactococcus*_b39c33	43
*Leuconostoc*_24cd1b	29
*Romboutsia*_464a52	27
*Enterobacteriaceae*_unclassified_031562	26
*Enterococcus*_028e2c	23
*Pediococcus*_462785	22
*Turicibacter*_dacd87	21
*Paeniclostridium*_69a95a	20
*Rhodococcus*_c292cc	19
*Sphingomonas*_471142	16
*Weissella*_6c2d2e	16
*Pseudomonas*_69d9bd	15
*Weissella*_6799a6	13
*Clostridioides*_3ca58b	12
*Clostridium_sensu_stricto*_1_c00514	12
*Pseudoclavibacter*_77b5e3	11
*Lactobacillus*_c1be91	10
*Sphingobacterium*_295e2f	10

of fermentation, as characterized by high abundance of *Lactobacillus* and higher content of organic acids.

Ammonia-N content is a measure of fermentation quality. In general, extensive fermentation during ensiling results in elevated ammonia-N content, with high-moisture silages having higher concentrations (Kung et al., 2018). In this study, silages from bunker and tower silos were positively associated with ammonia-N concentration (P = 0.055), likely caused by extensive fermentation, as illustrated by the higher content of lactic and acetic acid, and favored by lower DM content and thus higher moisture content (Table 2).

Additives, such as cultures of LAB or acid-based products (e.g., formic and propionic acid), are often used in ensiling to ensure silage quality by promoting a reduction in pH, thus inhibiting the activity of undesirable microorganisms and limiting loss of nutrients (McDonald et al., 1991). In the present study, the lowest pH was associated with forages produced with additives, but confounded with forages stored in bunker and tower silos. Although no significant chemical or microbial differences were found between bunker and tower silos in this study, the effect of silo type needs further investigation. For instance, Huffman et al. (2023) reported microbial differences between bunker and tower silos, noting that the relative abundance of the family Leuconostocaceae and pH were higher in tower silos. The dominance of the LAB genera *Lactobacillus*, *Weissella*, and *Leuconostoc* in the forages in this study was expected, considering that all were generally of high quality. The relative abundance of *Lactobacillus* was higher in additive-treated forage samples than in nontreated silage preserved as bales. A previous study by Franco et al. (2022) investigating the effect of silage treatments (i.e., without additive, with formic acid-based additive and with homofermentative LAB) on bacterial community composition and fermentation quality of grass silages found that *Lactobacillus* genus showed a strong positive correlation with total fermentation acids and a negative correlation with pH. Another study found that addition of LAB inoculant or formic acid treatment during ensiling of bur clover and annual ryegrass reduced bacterial diversity, enriched the relative abundance of *Lactobacillus* and resulted in improved silage fermentation (Li et al., 2019). A more recent study examined the epiphytic microbiota in Swedish grass-clover herbage and the effect of silage additive on fermentation profiles and bacterial community composition of the resulting silages and found some patterns, but the diverse microbiota in the herbage samples showed no clear connection to site, year, or harvesting time alone (Eliasson et al., 2023). Many studies have reported positive effects of using LAB inoculants based on (e.g., lactobacilli and pediococci as silage additives) but relatively few have investigated the effect of epiphytic LAB (e.g., *Leuconostoc* and *Weissella* species) on silage fermentation. Considering that the role of LAB inoculants is to ensure low pH and stability of the silage, genera *Weissella* and *Leuconostoc* may not necessarily contribute to improving silage quality. An investigation by Cai (1999) using strains of *Leuconostoc* and *Weissella* isolated from ley crops found that the strains were inhibited and stopped growing at pH below 4.5, thus allowing butyric fermentation by clostridia to occur. The growth pattern of these 2 heterofermentative strains was also reflected in our results, with both genera showing higher relative abundance in forage samples without an additive (Figure 5). The forages treated with acid or LAB inoculant likely experienced a more rapid pH drop, which probably inhibited growth of *Weissella* and *Leuconostoc*. The higher relative abundance of unclassified *Yersiniaceae* and *Lactococcus* in forage samples without additive treatment could also be pH-related. Eliasson et al. (2023) observed dominance of unclassified *Yersiniaceae* in some untreated silages, but high relative abundance also in some acid-treated grass-clover silages. Furthermore, the abundance of unclassified *Yersiniaceae* in that study showed positive correlations with pH and ammonia-N and negative correlations with formic and lactic acid and VFA (Eliasson et al. (2023). Bai et al. (2021) found that unclassified *Yersiniaceae* was associated with *Enterococcus faecalis*-inoculated alfalfa silage, resulting in higher pH than in other treatments. On characterizing the LAB population in whole-crop wheat ensiled with and without LAB inoculant (*Lactobacillus plantarum* ssp. *plantarum*), Ni et al. (2015) found that the inoculated silages had lower pH and higher lactic acid content. They also found that LAB in the spontaneously fermented silages mainly comprised heterofermentative species, including *Lacto-*

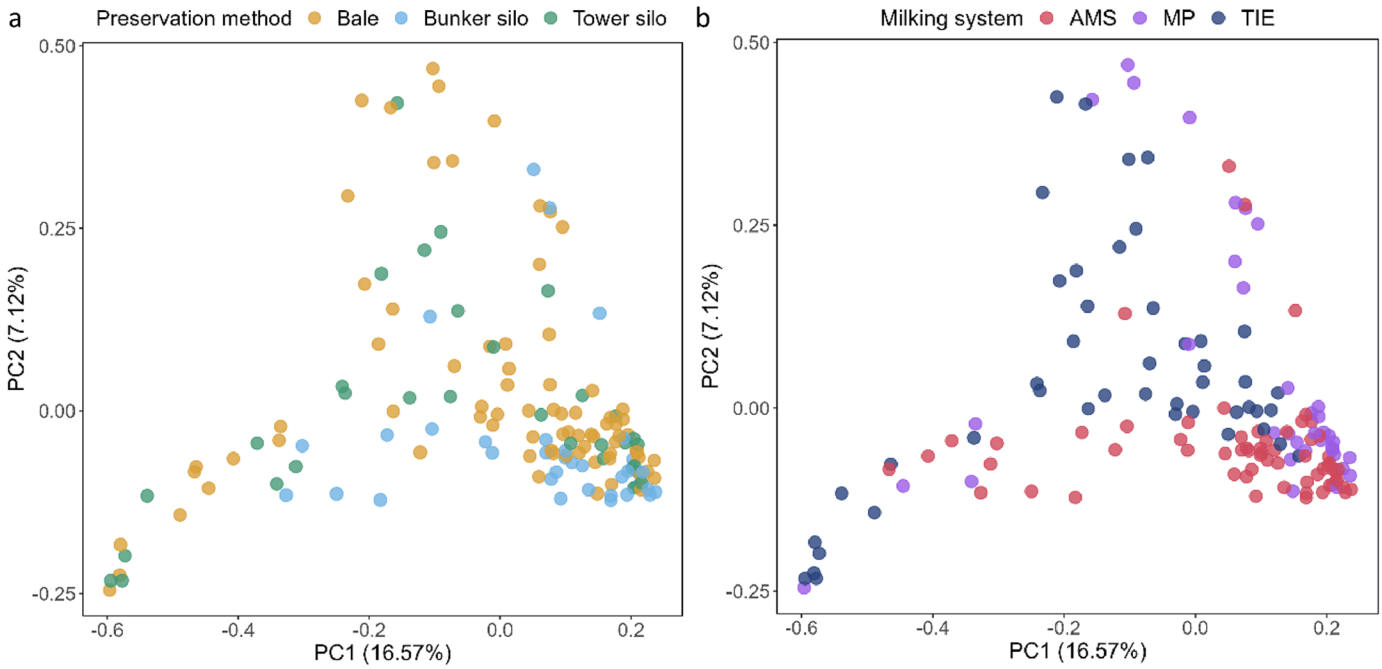

Figure 6. Principal coordinate (PC) analysis plot of microbiota in bulk tank milk samples from 3 sampling periods, analyzed using Bray-Curtis distance matrix, (a) colored by forage preservation method and (b) colored by milking system.

coccus, which did not grow well at low pH (pH < 4–4.5). Although these species grow vigorously and initiate lactic acid fermentation, lactobacilli play a more important role in promoting lactic acid fermentation for a longer time, leading to a sharp pH drop (Ni et al., 2015). In the present study, *Hafnia-Obesumbacterium* was associated with forages ensiled without additive (bales). Similarly, Zhao et al. (2021) found that *Hafnia-Obesumbacterium* was highly abundant in spontaneously fermented alfalfa silages, whereas the genus was concluded to accelerate protein degradation during ensiling of Italian ryegrass in a study by Wang et al. (2022). In line with these observations, in the study by Eliasson et al. (2023) the abundance of *Hafnia-Obesumbacterium* was positively correlated with pH and ammonia-N, and negatively with lactic acid, further confirming that presence of this genus in silages is undesirable.

The PCoA analysis (Figure 1a) and assessments of dominant ASV (Figure 2b) showed that the forage and bulk tank milk samples had a very low overlap of genera and ASV. To avoid overlooking potential transfer of bacteria from forage to milk, we searched for pairs where the same ASV was present in forage and the corresponding bulk tank milk sample. However, due to the short amplicon read length, it is important to keep in mind that even though the sample pairs had some ASV in common, it cannot be guaranteed that the same bacterium was present in both samples. No ASV was present in all sample pairs, but the ASV that appeared with the highest frequency in the paired samples was *Lactococcus*_b39c33 (Table 3). This was unsurprising, because Kelly et al. (2010) found some evidence that *Lactococcus lactis* isolated from the dairy environment may have evolved from plant-associated strains and speculated that adaptation to the dairy environment has resulted in loss of functions and acquisition of genes to facilitate growth in milk. Previous genome analysis of *L. lactis* has revealed that isolates from the dairy environment have higher capacity to bind to milk proteins (Tarazanova et al., 2017). In that study, which evaluated surface hydrophobicity, charge, emulsification properties, and attachment to milk proteins of 55 *L. lactis* strains, 3 proteins that altered surface hydrophobicity and attachment of milk proteins were identified. The results also showed that lactococci isolated from a dairy environment could bind higher amounts of milk proteins than plant isolates, with potential to alter starter culture functionalities (Tarazanova et al., 2017). In the present study, in addition to *L. lactis*, the genera *Leuconostoc*_24cd1b, *Pediococcus*_462785, *Sphingomonas*_471142 and *Weissella*_6c2d2e, all known as epiphytic bacteria and associated with herbage, were identified among the ASV pairs (Table 3). However, these genera are unlikely to survive the pasteurization process and may play a less important role in dairy products that rely on active non-starter LAB (e.g., long-ripened cheese from pasteurized milk). Surprisingly, despite the high abundance of *Lactobacillus* genus in forage samples, only one ASV of this genus, ASV c1be91, was found both in forage and milk,

and only in 10 out of the 50 sample pairs analyzed (Table 3). This suggests that forage was not the major source of *Lactobacillus* in bulk tank milk, which is important for long-ripened cheese. A similar conclusion was reached by Gagnon et al. (2020) in a study characterizing the microbiota of forages and bulk tank milk samples collected on 24 farms. They found that inoculation of *Lactobacillus buchneri* strain along with other LAB into forage had no effect on the prevalence and abundance of LAB in milk, and concluded that forage is only a minor contributor to LAB contamination of milk.

CONCLUSIONS

In this study, the microbiota detected in bulk tank milk was significantly different from the microbiota in forage samples. Use of additives had a large effect on forage microbiota composition, although it was confounded with method used for ensiling, making it impossible to distinguish effects of silage additive and ensiling method. Silages stored in bunker and tower silos (confounded with use of an additive) were associated with higher concentrations of acetic and lactic acid and *Lactobacillus*, whereas forage preserved as bales (confounded with no use of additive) was associated with higher content of DM and WSC, pH, yeast count, and abundance of the genera *Weissella*, *Leuconostoc*, and *Enterococcus*. The milking system was identified as the dominant factor affecting bulk tank milk microbiota. On analyzing common microbiota present in both forage and bulk tank milk at ASV level, the conclusion was that forage was not the major source of *Lactobacillus* found in bulk tank milk.

NOTES

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Nonstandard abbreviations used: AMS = automatic milking system; ASV = amplicon sequence variants; LAB = lactic acid bacteria; LA_rog = bacteria cultured on Rogosa agar; LA_sb = bacteria cultured on Slanetz-Bartley (S-B) agar; MP = milking parlor; OPLS-DA = orthogonal projections to latent structures discriminant analysis; P = period; PC = principal coordinate; PCA = principal component analysis; PCoA = principal coordinate analysis; PSV = phylogenetic species variability; S-B = Slanetz-Bartley agar; SLU = Swedish University of Agricultural Sciences; SNDHRS = Swedish national dairy herd recording scheme; TBC = total bacteria count; TIE = tiestall system; TRBC = thermoresistant bacteria count; WSC = water-soluble carbohydrates.

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