J. Dairy Sci. 107:10980–10997
https://doi.org/10.3168/jds.2024-25213
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Microbial communities in feed, bedding material, and bulk milk: Experiences from a feeding trial

T. Eliasson,[1,2]* L. Sun,[1] Å. Lundh,[1] H. Gonda,[3] A. Höjer,[2] K. Hallin Saedén,[2] and M. Hetta[4]
[1]Department of Molecular Sciences, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden
[2]Norrmejerier, SE-901 23 Umeå, Sweden
[3]Department of Applied Animal Science and Welfare, Swedish University of Agricultural Sciences, SE-750 07 Uppsala, Sweden
[4]Department of Applied Animal Science and Welfare, Swedish University of Agricultural Sciences, SE-901 83, Umeå, Sweden

ABSTRACT

There is an increasing interest in the microbiota of the dairy value chain, from field to fork. Studies to understand the effects of environmental, feed, and management factors on the raw milk microbiota have been performed to elucidate the origin of the bacteria and find ways to control the presence or absence of specific bacteria. In this study, we explored the microbiota in feedstuff, bedding material, and milk on a Swedish dairy farm to investigate the effects of feeding different silages on bacterial compositions throughout the dairy value chain. We evaluated 3 ensiling treatments: without additive, with acid treatment, and with inoculation of starter culture. The silage treatments were fed as partial mixed rations to 67 dairy cows for 3 wk each, with one treatment fed twice to evaluate if a potential change in milk microbiota could be repeated. The highest average total bacteria counts were found in the used bedding material (9.6 $\log_{10}$ cfu/g), whereas milk showed the lowest (3.5 $\log_{10}$ cfu/g). Principal coordinate analysis of the weighted UniFrac distance matrix showed clear separation between 3 clusters of materials: (1) herbage, (2) silage and partial mixed ration, and (3) used bedding material and milk. Surprisingly, the expected effect of the ensiling treatments on silage microbiota was not clear. Transfer of major bacteria from the silages and resulting partial mixed rations to the used bedding material was observed, but rarely to milk. The milk microbiota showed most resemblance to that of the used bedding material. *Lactobacillus* was a major genus in both feed and milk, but investigations at the amplicon sequence variant level showed that in most cases, the sequences differed between materials. However, low total bacteria count in the milk in combination with a high diversity suggests that results may be biased due to environmental contamination of the milk samples. Considering that the study was performed on a research farm, strict hygienic measures during the feeding experiment may have contributed to the low transfer of bacteria from feed to milk.

Key words: silage additive, silage microbiota, bulk tank milk microbiota

INTRODUCTION

There is an increasing interest in understanding the microbiota of the dairy value chain, from field to fork. Multiple studies have been conducted to explore microbial community composition in different environments and matrices, but also to determine the origin of milk microbiota (Ouamba et al., 2023). Specific attention has been devoted to nonstarter lactic acid bacteria (**LAB**), which are responsible for the formation of aroma components in many traditional cheeses (Bettera et al., 2023).

Lactic acid bacteria are found in a variety of ecological niches associated with dairy production, including forage crops and the resulting silages. Dairy production in Sweden is distributed throughout the country, and the botanical composition of forage leys varies between regions and farms. Ensiling is the most commonly used method to preserve forage crops in the Nordic countries, with LAB and water-soluble carbohydrates being crucial factors in making high-quality silage (Oliveira et al., 2017).

The microbiota in silage can be roughly divided into 2 groups: desirable and undesirable microorganisms. The desirable microorganisms are mainly LAB (e.g., *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Enterococcus*), which are epiphytic bacteria that occur naturally on forage crops and are important for the ensiling process. Undesirable microorganisms include *Clostridia*, *Enterobacteria*, and *Listeria*, as well as yeasts and molds

Received May 23, 2024.
Accepted July 10, 2024.
*Corresponding author: thomas.eliasson@slu.se

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

(Driehuis and Elferink Oude, 2000). Factors of great importance for the hygienic quality of silage include predrying and DM content of the herbage, as well as the use of additives of the right type and dose (Kung et al., 2003). Other risk factors include contamination by soil and its associated microbiota under wet harvesting conditions, swathing, and extended predrying of herbage in the field (Pahlow et al., 2003). In a recent study, we investigated the epiphytic microbiota in grass-clover herbages harvested at different sites and on different occasions in Sweden in order to explore the effects of different silage additives on the microbiota of the resulting silages (Eliasson et al., 2023). The results showed that the epiphytic microbiota in grass-clover herbage was not dependent on site per se, although major variation was observed between sites and harvesting occasions. Silage additives had a clear effect, although the most predictable and preferable silage from a microbial perspective resulted from inoculation with a LAB starter culture. Surprisingly, acid treatment with formic acid and propionic acid resulted in less preferable silages (Eliasson et al., 2023).

Microorganisms can spread in the local environment on dairy farms to the cow udder and finally to the raw milk via various pathways (e.g., feed residues, manure, and bedding material; Ouamba et al., 2023). To our knowledge, few previous studies have examined the impact of silage additives on the numbers and relative abundance (**RA**) of natural LAB associated with forages, and the flows of natural LAB through the dairy value chain. Ouamba et al. (2023) investigated the microbiota of different ration combinations and transfer rates of associated species to the raw milk, and found that silage-based forage rations shared more amplicon sequence variants (**ASV**) with the resulting raw milk than rations based on hay. They observed significant differences between milk samples associated with farms feeding different types of silage, but surprisingly, these differences were driven by *Enterobacteriaceae* and other *Proteobacteria*, rather than by LAB (Ouamba et al., 2023).

Our starting hypothesis was that the microbiota of the feed affects the microbiota of the raw milk. To minimize variation in other factors than feed which could have a confounding effect on the milk microbiota, the study was performed in a dairy research farm. In this way, in contrast to performing the study in commercial dairy farms, such factors could be controlled and kept more or less constant. The specific objectives of the study were to (1) explore the microbiota in different samples on a Swedish dairy farm (herbage, silage, partial mixed ration [**PMR**] and its ingredients, clean and used bedding material, and bulk milk) and (2) investigate the effects of feeding silages produced with and without ensiling additives on microbial communities throughout the dairy value chain, but particularly LAB.

MATERIALS AND METHODS

The experiment was conducted at Röbäcksdalen Research Centre in Umeå, Sweden (63.81°N, 20.23°E), which is part of Swedish Infrastructure for Ecosystem Science within the Swedish University of Agricultural Sciences (Uppsala, Sweden). Silages with different additives were made during June and July of 2020, and the feeding experiment was carried out from January to April of 2021. The full experiment is briefly described in the flowchart in Figure 1, with sampling points indicated.

Silage Production

The herbage used in silage making was cultivated on the research farm, in a 5-yr crop rotation with barley (*Hordeum vulgare*) in year 1; barley with an undersown forage mix comprising timothy (*Phleum pratense*), meadow fescue (*Festuca pratensis*), and red clover (*Trifolium pratense*) in year 2; and the forage mix as a ley cut 2 to 3 times per season in years 3 to 5. The soil type on the research farm is a silty loam with 2% to 5% clay, 3% to 6% OM, and a mean pH of 6.1, with textural properties identical down to 100 cm. The agronomic setting is typical for the northern Swedish coastal region and river valleys. The arable land on the farm (~200 ha) is divided into ~20 fields closely distributed around the dairy barn. More information on the agronomic conditions can be found in Zhou et al. (2019).

The silages were produced from the first (June 15–18) and second (July 24) cuts of the mixed grass leys. Actual cutting date was determined by phenological development of the crop, targeting forages with a high concentration of ME (≥11.0 MJ/kg DM). The leys were harvested with a disc mower conditioner, wilted in windrows aiming for a DM concentration of approximately 270 g/kg fresh matter (**FM**), and then precision-chopped to theoretical chop length of 16–32 mm. Three types of silages were produced: without additive (**UNTR**), with acid treatment (**ATR**), and with inoculation by starter culture (**INOC**). The ATR silage was produced with a formic and propionic acid-based additive (ProMyr NT-570, Perstorp, Sweden), added at a rate of 3 L/t FM. The INOC silage was produced with commercial LAB-based starter culture. However, due to shortage of supply from the manufacturer, 2 different starter cultures were used. The first batch of INOC, produced during the first cut, was inoculated with Feedtech Silage F10 (DeLaval, Tumba, Sweden), comprising a mixture of *Lactobacillus plantarum*, *En-*

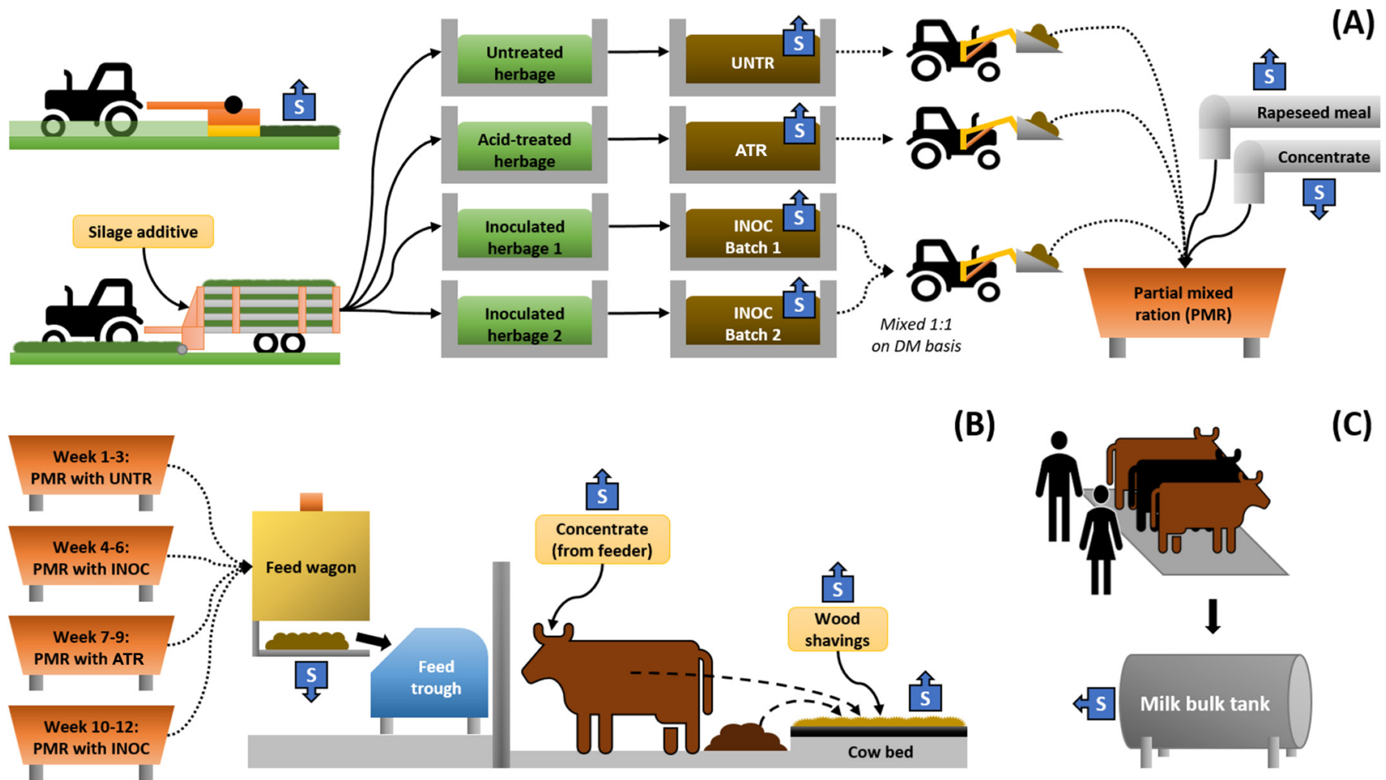

Figure 1. The experimental design, briefly summarized in 3 panels: (A) the ensiling process and preparation of partial mixed rations, (B) treatment schedule and animal housing, and (C) milk from last week of each treatment in bulk milk tank. Sampling points are marked with (S).

terococcus faecium, and *Pediococcus acidilactici*. The second batch of INOC, produced during the second cut, was inoculated with SiloSolve MC (Svenska Foder, Lidköping, Sweden), comprising similar bacteria except that *P. acidilactici* was replaced with *Lactococcus lactis*. Both INOC batches were prepared according to the instructions provided, with starter culture added at a rate of 2 L/t FM, resulting in the inoculation of 100,000 cfu/g for Feedtech Silage F10 and 250,000 cfu/g for SiloSolve MC. All silages were stored in separate bunker silos as described by Hetta et al. (2007). Details of silage chemical composition and hygienic quality are presented in Table 1.

Feeding Experiment Design

The feeding experiment was run for 12 wk, with each of the 3 silages evaluated for 3 wk (± 1 d). The order of treatments (**T**) was: (T1) UNTR, (T2) INOC, (T3) ATR, and (T4) INOC again. The INOC treatment was repeated to evaluate whether potential changes in milk microbiota were repeated. Each treatment was incorporated into a PMR that was fed to all animals included in the trial. The last week of each treatment was a sampling week in which data were recorded and samples were collected. On the day before the start of each treatment, the whole barn was thoroughly cleaned.

Animals and Diets

The experiment included ~67 (range 61–69) primi- and multiparous dairy cows (mainly Nordic Red). Average cow weight during the experiment was 653 kg (SEM 1.3 kg), and average milk production per cow was 32.8 L (SEM 0.12 L). The PMR were produced using one of the treatments (UNTR, ATR, or INOC), concentrate, rapeseed meal, and a mineral premix. When the INOC was fed, the 2 different batches were mixed 1:1 on a DM basis. The PMR was designed to meet the basic nutritional needs of dairy cows producing 25 kg ECM per day, and all PMR were set to be isocaloric (ME basis) and isonitrogenous. Additional concentrate was fed in proportion to milk yield. The PMR was fed through 30 feed bunks (Roughage Intake Control, Insentec B.V., Marknesse, the Netherlands) and additional concentrate through separate concentrate feeders. A stationary feed mixer (Nolan A/S, Viborg, Denmark) processed the PMR, which was delivered by automatic feeder wagons to the feed bunks

Table 1. Chemical composition and hygienic quality of the untreated (UNTR), acid-treated (ATR), and starter culture-inoculated (INOC) silages used in the feeding trial

Item	Silage (batch)			
	UNTR (first)	ATR (first)	INOC (first)	INOC (second)
pH	4.0	3.9	4.0	3.9
Lactic acid, g/kg DM	65.5	68.0	52.0	56.0
Acetic acid	18.0	13.5	15.5	17.0
Butyric acid	1.8	0.1	0.6	0.1
Nitrate	3.5	4.4	2.4	1.1
Yeast, log cfu/g	<2.0	<2.0	6.3	5.9
Mold	<2.0	<2.0	2.3	<2.0
Enterobacteriaceae	<2.0	<2.0	<2.0	<2.0
Escherichia coli	<1.0	<1.0	<1.0	<1.0
Aerobic spore-forming bacteria	<3.0	3.3	<3.0	3.7
Butyric acid spores[1]	<1.0	1.6	1.0	1.3
ME, MJ/kg DM	11.2	11.2	10.7	11.0
OM digestibility, %	77.0	77.2	73.4	76.3
DM, g/kg FM	277	306	320	280
NDF, g/kg DM	480	457	524	439
ADF	285	270	314	275
Crude fat	41	39	39	39
Water-soluble carbohydrates	15	18	16	29
Ash	77	77	69	85
CP	169	172	155	146
Soluble CP, g/kg CP	670	545	600	561
Ammonia-N, g/kg N	109	80	97	84

[1]Spore-forming bacteria that produce butyric acid.

6 times per day. The amount of feed delivered was monitored daily to avoid excessive leftovers. Detailed information on animals, feed intake, and milk production is provided in Table 2.

Housing and Milk Collection

The dairy barn where the experiment took place was insulated, with a controlled indoor temperature at 10 to 15°C. The cows were kept loose-housed in 2 aisles, one for eating and one for resting with cubicles and a rubber mattress for each cow bed. The cubicles were manually cleaned with a scraper each day and covered with wood shavings (pine and spruce) on a daily basis to keep the animals dry. The cows were milked twice daily in a milking parlor (2 × 8) at 0600 h and 1600 h. The milking procedure comprised (1) udder wiping with clean wet cloth, (2) drying with clean dry paper, (3) premilking by hand, and (4) applying the milk liners. Individual milk production was recorded daily using gravimetric milk recorders (S.A. Christensen & Co., Kolding, Denmark). The milking equipment and the milking parlor were thoroughly cleaned and washed after each milking, and the milk was collected and transported to the dairy every second day.

Sampling and Sample Preparation

Herbage. The botanical composition of the ley from each individual field was evaluated just before harvest using the dry-weight ranking method developed by Mannetje and Haydock (1963). In short, the leys were assessed by walking across the field at 15-m intervals, with up to 30 observations per field using a 1 m^2 steel quadrat to assess the areal contribution of the major plant species.

Fresh herbage samples for estimation of total bacteria count and microbial community analysis were taken

Table 2. Production averages for the cows during feeding of the untreated (UNTR), acid-treated (ATR), and starter culture-inoculated (INOC) silage treatments

Item	T1-UNTR	T2-INOC	T3-ATR	T4-INOC	SEM
Days, n/treatment	22	20	22	21	
Animals					
Cows, n/d	63.1	68.2	68.9	69.0	0.29
Weight,[1] kg/cow	639	655	651	653	2.4
Feed intake[2]					
Total, kg DM/d	20.2	21.3	21.1	20.9	0.09
PMR	14.5	14.9	15.3	15.7	0.07
Silage	10.2	10.0	10.9	10.7	0.05
Concentrate	3.4	3.3	3.5	3.6	0.02
Rapeseed meal	0.8	1.4	0.8	1.3	0.01
Concentrate	5.6	6.1	5.1	4.5	0.06
Test milking[3]					
Milk, kg	29.8	32.2	32.4	30.6	0.48
ECM, kg	32.8	34.6	35.7	33.1	0.47
Fat, %	4.60	4.37	4.58	4.45	0.065
Protein, %	3.77	3.79	3.81	3.75	0.049
Urea, mmol/L	5.0	4.9	4.6	5.0	0.09
SCC, 1,000 cells/mL	125	115	147	125	12.4

[1]Weight was recorded ~2 times per cow during the last week of each treatment.
[2]Feed intake was recorded daily for each cow during the last week of each treatment.
[3]Test milking was performed during 2 consecutive milking occasions in the last week of each treatment.

from every field directly after cutting. Grab samples (~15 kg FM) were taken evenly with sterile nitrile gloves from the herbage swaths in each field and placed in plastic bags. The herbage sampled from each field was mechanically chopped into smaller pieces and mixed thoroughly before further processing.

Feeds and Bedding Material. Samples of silage, concentrate, and rapeseed meal were collected for determination of DM at least once every week throughout the experiment to maintain the correct mixing proportions in the PMR. Drill core samples from the bunker silos designated for analysis of chemical composition and hygienic quality were taken by Eurofins Agro Testing (Kristianstad, Sweden) ~3 mo after the silos were closed (2 mo for the second INOC silo). The drill cores were taken from each silo by drilling from top to bottom in an evenly distributed pattern.

All sampling for estimation of total bacteria count and microbial community analysis was performed during the last week of each treatment. Silage, PMR, and used bedding material were sampled 3 times (every second day). Concentrate, rapeseed meal, and wood shavings were sampled once (mid-week). Silage was sampled from the opened bunker silos by grab sampling with sterile nitrile gloves at a minimum of 20 evenly distributed spots over the open surface immediately after silage was taken out. The PMR was sampled by grab sampling with sterile nitrile gloves from the outlet of the feeder wagon during one full filling round of the feed bunks. The silage and PMR samples were ground with a sanitized compost grinder before further processing. The used bedding material was sampled by taking grab samples with sterile nitrile gloves from the bottom half of every second cubicle, giving a sample comprising a mixture of wood shavings, manure, and various animal fluids. Concentrate and rapeseed meal were sampled from both the concentrate feeders and the individual lines going to the mixer. Sampling was performed by releasing a minimum of 5 kg from each source into a plastic bag. Clean wood shavings were sampled with sterile nitrile gloves from the most recently used bunker silo (2 in total) by grab sampling at a minimum of 20 evenly distributed spots on the open surface (top layer discarded).

Milk. Milk samples for microbial community analysis were sampled from the bulk tank in the morning on the same days that the silage, PMR, and used bedding material were sampled. On these occasions, the bulk tank contained milk from 4 milkings, 2 d of morning milk and 2 d of evening milk. Samples (40 mL) were drawn into duplicate sterile Falcon tubes (50 mL; Sarstedt) and immediately stored frozen (−20°C). At the end of each sampling week, all milk samples were transferred to storage at −80°C. Additionally, a sample for estimation of total bacteria count was taken by Norrmejerier (Burträsk, Sweden) after transportation of the milk to the dairy (within 2 h from collection in the barn). Finally, test milking was performed during the last week of each treatment by measuring the yield and sampling the milk of each cow on 2 consecutive milking occasions (afternoon and morning).

Analytical Methods

Feed Composition. The chemical composition of silage was analyzed with near-infrared spectroscopy by Eurofins Agro Testing (Wageningen, the Netherlands). An unspecified internal method was used for DM and ash, and no methods were specified for nitrate, butyric acid, and ADF content. The ME content was calculated from the chemical composition by Eurofins Agro Testing (Kristianstad, Sweden). The hygienic quality of the silages was analyzed by Eurofins Food and Feed Testing (Jönköping, Sweden). The methods used were as follows: unspecified for pH, NMKL 98 for yeast and mold (NMKL, 2005), AFNOR 3M 01/06-09/97 for *Enterobacteriaceae*(ISO, 2017), AFNOR 3M 01/08-06/01 for *Escherichia coli* (ISO, 2001), and internal method 7 for spore-forming aerobic bacteria and butyric acid spores.

Weekly in-barn analysis of DM in silage, concentrate, and rapeseed meal was performed by oven-drying samples at 60°C to constant weight. Chemical composition of concentrate and rapeseed meal in each batch delivered was analyzed by the producer Lantmännen (Umeå, Sweden).

Milk Composition. Samples from test milking were analyzed by Eurofins Milk Testing (Jönköping, Sweden). Milk composition was analyzed with mid-infrared spectroscopy (Fourier Transform Infrared, FOSS, Hilleröd, Denmark) and SCC with fluorescence-based cell counting (Fossomatic, FOSS, Hilleröd, Denmark).

Estimation of Total Bacteria Count. Culturing of bacteria was performed directly after sampling and sample preparation for all materials except milk. Each sample was mixed thoroughly, and 2 subsamples of 30 g each were placed in stomacher bags, mixed with 270 g peptone water (1 g/L Oxoid Peptone Bacteriological, Thermo Scientific), and run in a stomacher (Stomacher 400, Seward) for 1 min. A 10-mL subsample from each bag was pipetted into a sterile glass vial, and a dilution series was performed with peptone water, followed by spread-plating of selected dilutions (0.1 mL/plate). For lactobacilli, de Man, Rogosa, and Sharpe (**MRS**) agar (54.6 g/L MRS agar, Merck) was used. For total bacteria, a modified (0.08 g/L Delvocide, DSM) milk plate count agar (**MPCA**; 19.5 g/L MPCA agar, Liofilchem) was used. The MRS plates were placed inverted in sealed jars with anaerobic medium, and the MPCA plates were stacked in perforated plastic bags. All plates were incu-

bated in a heating cabinet at 30°C for 48 h. For milk, estimation of total bacteria count was performed by Norrmejerier (Burträsk, Sweden) according to an internal protocol with plate count agar and incubation at 30°C for 72 h.

Microbial Community Analysis. For all materials except milk, preparation took place in connection with estimation of total bacteria count. Two additional subsamples of 30 g each were placed in stomacher bags and mixed with 270 g of 1/4 strength Ringer's solution with 0.5 mL/L Tween 80 (Merck), prepared according to O'Brien et al. (2007), and run in a stomacher (Stomacher 400, Seward) for 1 min. A 50-mL subsample from each bag was pipetted into a sterile Falcon tube and run in a centrifuge at 7,000 × *g* and 10°C for 25 min. After centrifugation, the tubes were decanted without losing any pellet, and refilled to the 10-mL mark with Ringer solution. The tubes were vortexed until the pellet was dissolved and 1 mL from each was pipetted into a 2-mL cryo-tube. The cryo-tubes were frozen at −80°C until DNA extraction was performed, which was done as described in Eliasson et al. (2023).

The 50-mL Falcon tubes containing milk samples were thawed in a water bath at 25°C for 1 h. The thawed milk was then carefully mixed by inverting the tubes by hand a few times, and 1.8-mL subsamples were pipetted into 2-mL collection tubes provided with the PowerFood DNA isolation kit (Qiagen AB, Sollentuna, Sweden). This step was followed by the customized protocol described in Sun et al. (2023). Random samples were checked with a Nanodrop spectrophotometer (Thermo Fisher Scientific) to ensure DNA extractions with sufficient yield and quality. Finally, the bacterial DNA was stored at −80°C until further analysis.

Library Construction and Sequencing

The bacterial DNA was sent to Novogene (Cambridge, United Kingdom) for library construction and sequencing. An initial quality control of the DNA was performed by agarose gel electrophoresis. The V4 region of the 16S rRNA gene was amplified using the primers 515f (GTGBCAGCMGCCGCGGTAA) and 805r (GACTACHVGGGTATCTAATCC), and a library was constructed. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Sequencing was performed on the Illumina NovaSeq PE250 platform (50k tags per sample). The raw reads were de-multiplexed before delivery. From the initial 184 samples sent to Novogene, all passed the quality control. The raw sequencing data were deposited in the Sequence Read Archive at the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/sra), under accession number PRJNA1155694.

Bioinformatics

Bioinformatic data processing was performed using QIIME 2 2022.11 (Bolyen et al., 2019). The raw demultiplexed reads were trimmed with Cutadapt to remove primer sequences (Martin, 2011), and all reads containing nonidentified bases or missing primer sequences were removed. Further trimming, de-nosing, de-replication, read merging, and removal of chimeras were performed with DADA2 (Callahan et al., 2016). Truncation length was set to 160 bp for forward reads and 146 bp for reverse reads, as it gave the best read recovery after testing different levels of truncation. Phylogenetic trees were built using FastTree and MAFFT (Price et al., 2010; Katoh and Standley, 2013). Alpha and β-diversity were estimated, and principal coordinate analysis (**PCoA**) was performed using the q2-diversity plugin. Faith's phylogenetic diversity index (**FPDI**; Faith, 1992) was used to compare diversity, and weighted UniFrac distance matrix (Lozupone et al., 2007) and PCoA results were used to compare microbiota composition between and within materials. Taxonomy was assigned to ASV with q2-feature-classifier (Bokulich et al., 2018) using release 138 from the Silva database (Quast et al., 2013) as reference. For ASV with higher RA not passing species annotation by QIIME2, selected ASV were elaborated further using Nucleotide BLAST and the 16S ribosomal RNA sequences database as reference (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed Feb. 28, 2024), where only hits with 100% query cover and identity were considered (Zhang et al., 2000).

Statistical Analysis

The raw output files (.qza) from QIIME 2 were imported to R with the *qiime2R* package (Bisanz, 2018) together with all other data. Tables and diagrams were produced with R 4.3.1 (R Core Team, 2021), using the *tidyverse* package (Wickham et al., 2019). Statistical evaluation was performed with the additional packages *car* (Fox and Weisberg, 2019) and *emmeans* (Lenth, 2024).

Individual daily records of cow weight, feed intake, and milk production were first checked for outliers by using the z-score method for each variable and cow. Values deviating by ≥3 SD from the mean were discarded. Data from the last week of each treatment were filtered out and arithmetic means were calculated for each treatment together with SEM for all treatments, for each variable. The test milking results were filtered to only include complete records (i.e., cow data with missing values in any of the measured variables were discarded). Arithmetic means were calculated for each treatment and SEM for all treatments, for each variable. Compositional variables (fat, protein, urea, and SCC) were related to milk volume before calculation of treatment means and SEM.

Table 3. Total bacteria and lactobacilli counts ($\log_{10}$ cfu/g) in the different materials during feeding of the untreated (UNTR), acid-treated (ATR), and starter culture-inoculated (INOC) silage treatments[1]

Type	Material	T1-UNTR	T2-INOC	T3-ATR	T4-INOC	SEM	*P*-value
Total bacteria[2]	Silage	OG	7.3	6.4	7.4	0.12	0.001
	Concentrate	5.2	4.4	4.6	4.6	0.09	0.005
	Rapeseed meal	6.0	4.3	5.3	5.2	0.16	0.001
	Partial mixed ration	8.4[3]	7.7	6.8	8.0	0.12	<0.001
	Wood shavings	5.9	5.7	6.4	6.3	0.13	0.153
	Used bedding material	9.6	9.4	9.5	9.6	0.03	0.023
	Milk[4]	3.5	3.6	3.5	3.6	0.09	0.952
Lactobacilli[5]	Silage	9.2	8.8	8.6	8.7	0.03	<0.001
	Concentrate	3.5	3.3	3.9	3.3	0.14	0.611
	Rapeseed meal	5.4	3.9	3.8	NA	0.21	<0.001
	Partial mixed ration	9.0	8.8	8.4	8.6	0.04	<0.001
	Wood shavings	4.1	4.3	4.6	5.1	0.12	0.003
	Used bedding material	8.0	8.0	7.9	8.2	0.06	0.413

[1]OG = overgrown by unknown microorganism, NA = no colonies on plates.

[2]Aerobic incubation on modified milk plate count agar (0.08 g/L Delvocide, DSM).

[3]Counting only possible on one plate.

[4]Aerobic incubation on plate count agar.

[5]Anaerobic incubation on de Man, Rogosa, and Sharpe agar.

For evaluation of total bacteria count, arithmetic means were calculated for each material and treatment. A one-way ANOVA was performed for each material to evaluate the effect of treatment. For materials with a significant treatment effect, 2-tailed Welch *t*-tests were performed on all treatment combinations, and *P*-values were adjusted to avoid falsely rejected hypotheses according to Benjamini and Hochberg (1995), with $P < 0.05$ considered significant.

For α-diversity (measured as FPDI), a boxplot was produced for evaluation of differences between materials and treatments. Arithmetic means and SEM were calculated, and 2-tailed Welch *t*-tests were performed (as described above). Microbial composition was evaluated by pooling the reads by technical replicates (n = 2), followed by rarefication at the lowest sampling depth found in the data set (28,806 reads/sample). Arithmetic means were calculated to the levels described in the diagrams, and data were evaluated descriptively. Taxa found below 0.1% RA (29 rarefied reads) were considered as detected, but not as clear findings. For evaluation of treatment effects, quasi-Poisson regression and pairwise comparisons with Tukey adjustment were performed per genus or ASV.

RESULTS

Bacterial Enumeration, Composition, and Diversity

The bacterial enumeration on different media showed major variation between materials (Table 3). The highest average number of total bacteria was found in used bedding material (9.6 $\log_{10}$ cfu/g), whereas the lowest average was found in milk (3.5 $\log_{10}$ cfu/g), with both these differing significantly from all other materials. The mean number of total bacteria in silage and PMR (7.1 and 7.5 $\log_{10}$ cfu/g) was different from that in all other materials and from each other. The highest average number of lactobacilli was found in silage and PMR (8.8 and 8.7 $\log_{10}$ cfu/g, respectively), with both differing from the other materials. Among the silages, ATR had lower number of total bacteria than both batches of INOC, whereas UNTR had higher number of lactobacilli than the other silages. In PMR, differences in both total bacteria and lactobacilli were found between all silage treatments, except between the first and second INOC batches. Herbage was only randomly evaluated for lactobacilli during the first cut (mean 4.3 $\log_{10}$ cfu/g, n = 26, SEM = 0.10).

The PCoA of the weighted UniFrac distance matrix explained 68.4% of the variation in bacterial composition by the first 3 principal coordinates (Figure 2). The PCoA plot revealed rather clear separation between 3 clusters of materials: (1) herbage, (2) silage and PMR, and (3) used bedding material and milk. Concentrate, rapeseed meal, and wood shavings were more spread in the PCoA plot, but were still rather separated from all other materials. Thus, the microbiota of milk was closest to that of used bedding material.

Alpha diversity of the microbiota, measured as FPDI, varied widely between the materials, and to some extent also between the treatments (Figure 3, herbage excluded). Milk had the highest average FPDI (70.2), followed by used bedding material (44.1) and wood shavings (41.1). The FPDI of milk was different from that of all other materials, whereas used bedding material differed from all other materials except wood shavings. The lowest average FPDI was found in herbage (22.2, SEM 1.34), followed by silage (24.2), PMR (27.7), concentrate

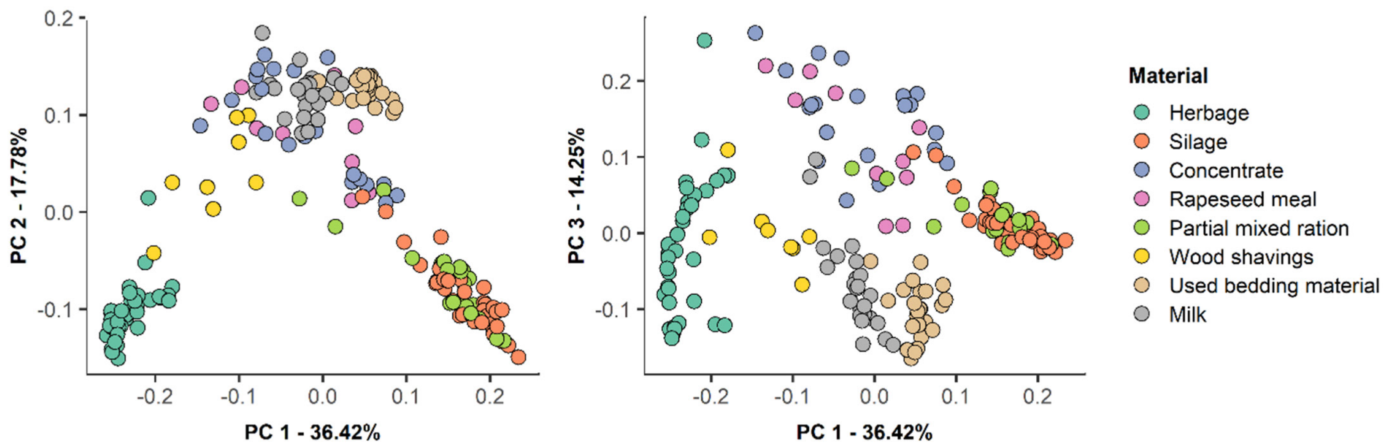

Figure 2. Principal coordinate analysis plots of the weighted UniFrac distance matrix of the microbiota of the different materials. The diagrams include sample replicates and show the first 3 principal coordinates (PC) and their contribution to the total variation in microbiota.

(32.9), and rapeseed meal (33.6). Herbage differed from all other materials except silage. Silage FPDI differed from that of PMR, concentrate, rapeseed meal, and wood shavings. The FPDI of PMR differed from that of wood shavings, but not from that of concentrate and rapeseed meal. In terms of FPDI, concentrate and rapeseed meal did not differ from each other or from wood shavings.

Herbage and Silage Composition

Evaluation of the botanical composition of herbage (Supplemental Table S1, see Notes) showed a major proportion of timothy (mean 70%, range 53%–90%), whereas other plant species varied to a larger extent. Dandelions were mainly found in herbage J (21%), whereas annual bluegrass was mainly found in herbage F (27%). Herbage F was also the only herbage with tufted hairgrass (8%). Red clover was found in a high proportion in herbage L (21%), whereas white clover was only found in herbage C (13%). Herbage C also contained a high proportion of meadow fescue (16%). The proportions of other forage species varied from 3% to 15% in first-cut herbages and from 21% to 41% in second-cut herbages.

The top 30 bacterial genera in herbages and the corresponding silages are presented in Figure 4. The second cut of herbages and the corresponding second INOC batch was not initially planned, but was necessary because the first INOC batch was not sufficient for the

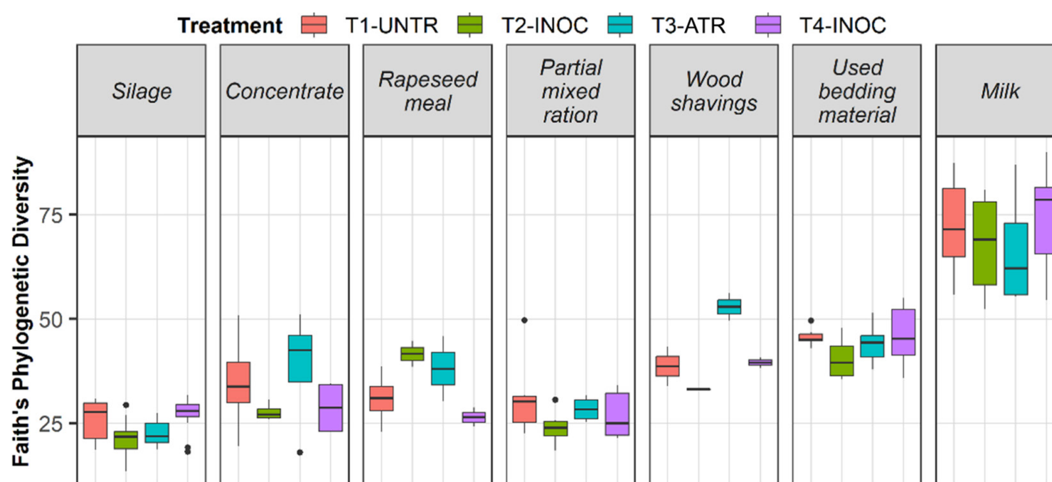

Figure 3. Boxplots of α diversity, estimated as Faith's phylogenetic diversity index, of the microbiota in feedstuffs, bedding material, and milk during the untreated (UNTR), acid-treated (ATR), and starter culture-inoculated (INOC) silage treatments in the feeding trial. The central box represents the interquartile range (IQR), encompassing the middle 50% of the data. The line inside the box indicates the median. Whiskers extend from the box to the smallest and largest values within 1.5 times the IQR from the first and third quartiles. Data points outside this range are shown as individual outliers (dots).

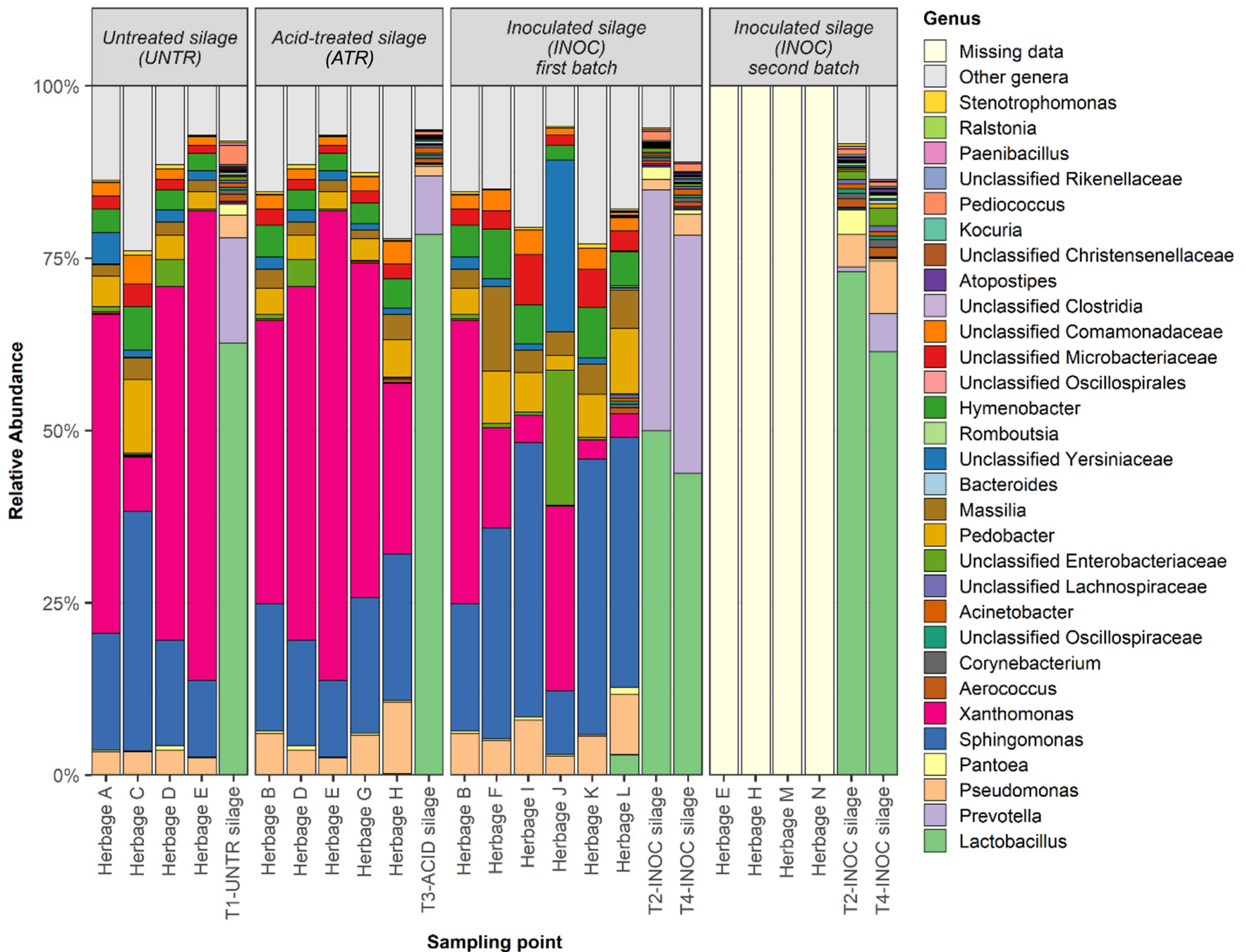

Figure 4. Relative abundance of the top 30 bacterial genera in the harvested herbages, and in the corresponding silages (sampled during each treatment). Figure represents sequence data pooled by technical replicate (n = 2). Silages were fed in the following order: (T1) UNTR, (T2) INOC, (T3) ATR, and (T4) INOC. The INOC silages were mixed 1:1 on DM basis during the T2-INOC and T4-INOC treatments.

feeding experiment. Due to a lack of communication, herbage was not sampled during the second cut. First-cut herbages showed a varying microbiota, with *Xanthomonas* and *Sphingomonas* contributing most to RA (mean 28.3% and 24.4%, respectively). Herbage J had high RA of unclassified *Yersiniaceae* (25.0%) and unclassified *Enterobacteriaceae* (19.6%). Other genera present in higher average RA were *Pedobacter* (5.4%), *Pseudomonas* (5.4%), *Hymenobacter* (4.5%), and *Massilia* (3.7%), with mostly minor variation between herbages. The genus *Lactobacillus* was barely detected, except in herbage L, which was also the only herbage in which *Aerococcus* and *Corynebacterium* were found. The most abundant genera observed in the herbages were barely present in the corresponding silages.

The silage microbiota mainly comprised the 3 genera *Lactobacillus*, *Prevotella*, and *Pseudomonas*. *Lactobacillus* was found in average RA of 61.6% (range 43.8%–78.4%), *Prevotella* in RA of 16.6% (range 0.7%–34.9%), and *Pseudomonas* in RA of 3.6% (range 1.4%–7.6%). Despite the major variation in these genera between silages, pairwise comparisons revealed no significant differences. However, differences were found for *Pediococcus*, which was present in higher RA in UNTR than in the other silages.

Microbiota of the Different Materials

The top 30 bacterial genera in all materials (except herbage) by treatment are presented in Figure 5. In

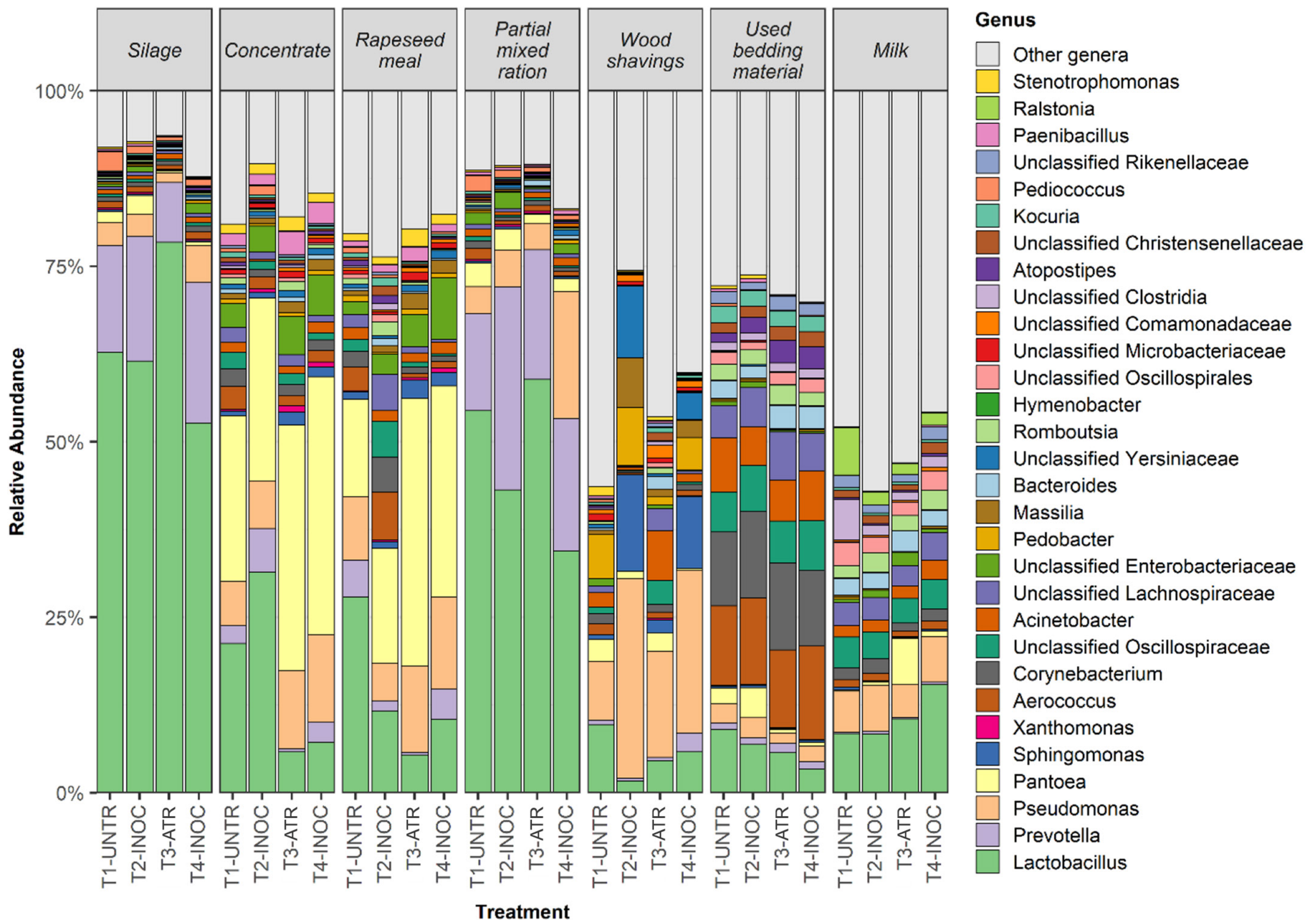

Figure 5. Relative abundance of the top 30 bacterial genera in feedstuffs, bedding material and milk during the last week of each treatment in the feeding trial. Figure represents sequence data pooled by technical replicate (n = 2). The treatments were: untreated silage (T1-UNTR), inoculated silage (T2-INOC), acid-treated silage (T3-ATR), and a repeat of inoculated silage (T4-INOC).

general, we found little variation between treatments in microbiota within the different materials during the feeding trial. The RA in the different silages in Figure 5 is the same as in Figure 4, with the exception of INOC, which in Figure 5 is illustrated as the pooled value (1:1) of those 2 silage batches. For ATR, higher RA of *Lactobacillus* was observed in the silage, but the RA was not significantly different from that in the other silages. Concentrate and rapeseed meal showed similar microbiota and 3 main genera were observed at high average RA, namely *Pantoea* (30.4% and 24.6%, respectively), *Lactobacillus* (16.4% and 13.8%, respectively), and *Pseudomonas* (9.2% and 9.9%, respectively). The microbiota of the PMR showed the strongest resemblance to that of the silages, despite the high inclusion of concentrate and rapeseed meal (Table 2). However, the number of genera with average RA >0.1% increased from 43 in the silages to 58 in the PMR. The 3 main genera in silage, i.e., *Lactobacillus*, *Prevotella*, and *Pseudomonas*, were observed at 47.8%, 20.0%, and 7.7% average RA, respectively, in PMR. As seen for the silages, the RA of *Pediococcus* was significantly higher in the PMR containing UNTR than in the other PMR. A numerically high RA of *Pseudomonas* (18.1%) was observed in the PMR during the second INOC treatment, but RA was not significantly different from that in the other materials.

Wood shavings showed generally high average RA of *Pseudomonas* (18.8%). This genus and the genera *Sphingomonas*, unclassified *Yersiniaceae*, and *Massilia* showed a tendency to be present in higher RA in the INOC treatments. However, due to the low number of sampling occasions, this was not further evaluated. Additionally, the genera *Cellvibrio* and *Glutamicibacter* were found at higher RA in the wood shavings, but were not among the top 30 genera (shown in Figure 5). Used

bedding material contained many genera, but none was clearly dominant, and it showed little resemblance to the wood shavings. The genera present in highest average RA were *Aerococcus* (12.0%) and *Corynebacterium* (11.5%), followed by *Acinetobacter* (6.5%), *Lactobacillus* (6.3%), unclassified *Oscillospiraceae* (6.3%), and unclassified *Lachnospiraceae* (5.6%).

Raw milk was the most diverse of all materials (Figure 3), comprising a total of 122 genera with average RA >0.1%. Thus microbial diversity was much higher than in used bedding material (81 genera) or PMR (58 genera). The highest average RA was recorded for *Lactobacillus* (10.7%, range 8.4%–15.5%), with a tendency for increasing RA over the course of the experiment, but with no significant difference between treatments. This was followed by *Pseudomonas*, with average RA of 5.9%. The only significant difference in milk between treatments was found for unclassified *Clostridia*, which was present in higher RA when feeding the UNTR compared with the other treatments.

Most Abundant ASV Found During the Feeding Trial

To further evaluate the flow of bacteria from feed to milk, an investigation on ASV level was performed. In total, 15,766 ASV were detected in PMR, used bedding material, and milk. Of these, only 151 were found at average RA >0.1%, and only 15 at average RA >1.0%. The top 50 ASV were selected based on their average RA in all 3 materials, and a heatmap was produced (Figure 6). The overall finding was that the most abundant ASV in PMR were to some extent also present in used bedding material, but rarely in the milk. However, several ASV which were abundant in used bedding material were also abundant in the milk.

Effect of Silage Additive

At the genus level, only a minor effect of additive was observed in the resulting silages. The microbiota of the silages was reflected in the corresponding PMR, but closer investigation on ASV level was performed to evaluate whether the silage additives separated the treatments. Among the 4 species of bacteria included in the starter cultures used for INOC, only *Lactococcus lactis* was found among the top 50 ASV in the PMR (Figure 6). However, this ASV could represent another strain, and the other bacteria in the starter culture could be among the unidentified ASV. The ASV in PMR showing the highest RA were *Lactobacillus acetotolerans* (e1910) with an RA range of 20.4% to 36.0% and *Prevotella* (f74b4) with a range of 13.0% to 27.5%. Tendencies for differences between treatments were observed, but none of these were significant.

Among the remaining top 50 ASV, only a few showed significant differences between treatments. *Lactobacillus* (03f2f) was less abundant in PMR in the ATR treatment than in the UNTR and the first INOC treatments. The RA of the ASV *Lactobacillus* (1d194) was significantly lower during the second INOC treatment compared with UNTR. *Lactobacillus* (6b62e) was mainly present during the UNTR treatment. The RA of *Lactobacillus buchneri* (dc9d7) was significantly lower during the ATR treatment compared with UNTR and the first INOC. *Lactobacillus fructivorans* (08c5f) was mainly present during the ATR treatment, and at a notably higher RA (16.7%). The RA of *Pediococcus* (3d185) was higher during UNTR than in the other treatments, whereas unclassified *Enterobacteriaceae* (8622f) was less abundant during the ATR treatment compared with UNTR. Only a few ASV showed strong tendencies for higher abundance during one of the treatments, such as *Prevotella paludivivens* (5e89c) during the ATR treatment and *Pseudomonas* (15a37 and 3ae60) during the second INOC treatment. However, these differences were not significant, as 1 of the 3 sampling occasions typically contributed to the high average RA for a certain treatment.

Similarities Between PMR and Used Bedding Material

Lactobacillus acetotolerans (e1910), *Prevotella* (f74b4), and other highly abundant ASV in the PMR were also found in used bedding material, but at lower RA. In addition, ASV which were rarely found or only found at a low RA in PMR were found to be part of the microbiota in used bedding material. The most abundant ASV in used bedding material were *Aerococcus* (0cb4d) and *Acinetobacter* (8572d), with average RA of 10.6% and 5.9%, respectively. Further, a group of *Corynebacterium* ASV were observed, at a total average RA of 8.8%.

A few significant differences between treatments were found for used bedding material. *Lactobacillus* (1d194 and 6b62e) and *Pediococcus* (3d185) showed higher RA during the UNTR treatment, whereas *Paeniclostridium* (0bb17) and *Turicibacter* (62c62) showed their highest RA during the ATR treatment. *Romboutsia* (8f04c) showed higher RA during the ATR treatment than during the second INOC treatment. Unclassified *Enterobacteriaceae* (8622f) showed higher RA during the first INOC treatment compared with both the ATR and second INOC treatments. Unclassified *Lachnospiraceae* (a411d) showed higher RA during the ATR treatment compared with the second INOC treatment.

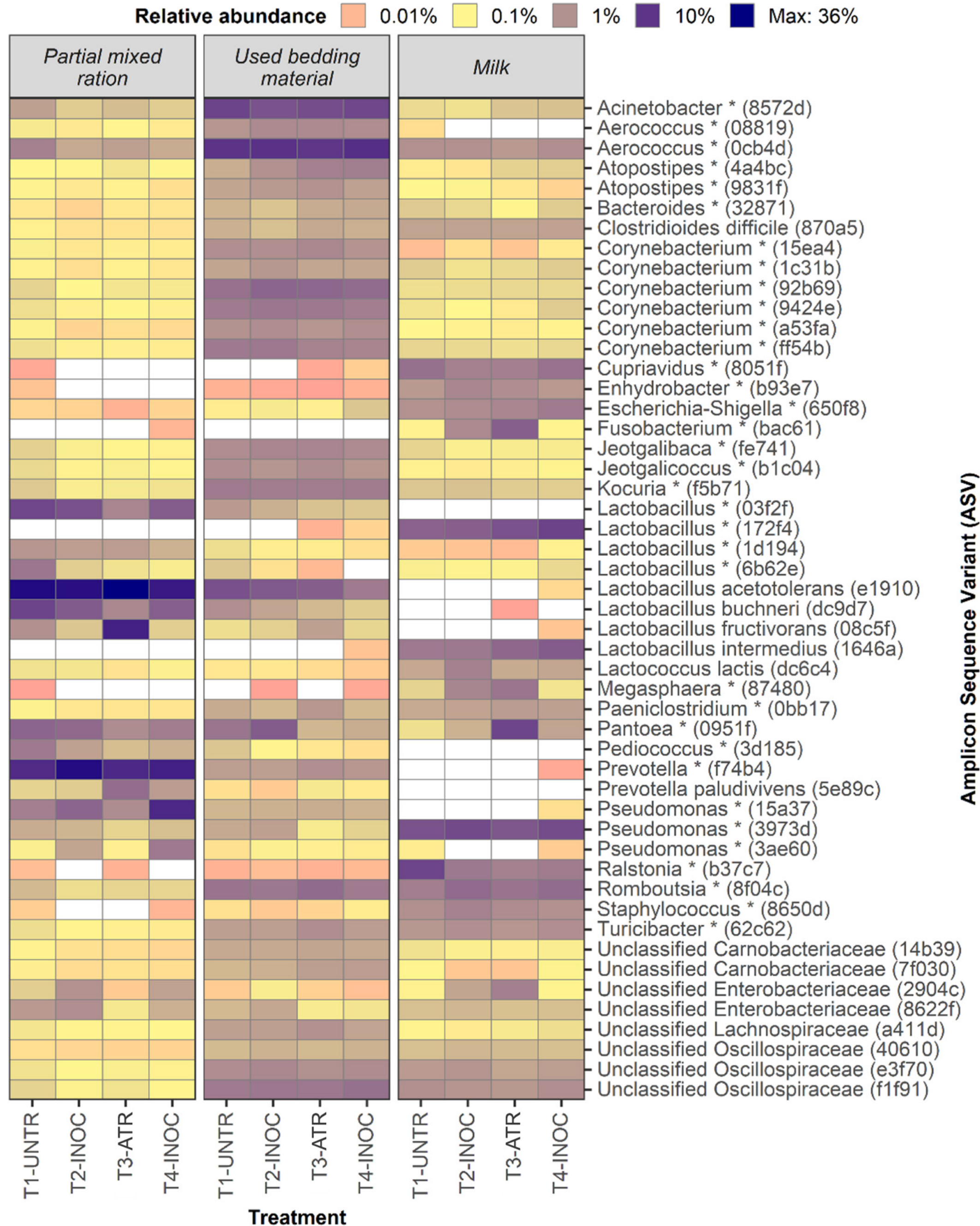

Figure 6. Heatmap showing $\log_{10}$-transformed relative abundance (RA) of the top 50 ASV found in partial mixed ration, used bedding material, and milk during the feeding trial. The treatments were: untreated silage (T1-UNTR), inoculated silage (T2-INOC), acid-treated silage (T3-ATR), and a repeat of inoculated silage (T4-INOC). The legend scale was converted back to RA and nonpresent ASV were colored white for easier interpretation. *Indicates that the ASV was not classified further than the genus level. Max = maximum.

ASV in Milk and Their Potential Origin

The only ASV present in high RA in all 3 materials were *Aerococcus* (0cb4d) and *Pantoea* (0951f), with the latter also showing a tendency for treatment differences (not significant). The most abundant ASV in milk was *Pseudomonas* (3973d), at an average RA of 5.1%, and this ASV was also found in the other materials, although at lower RA. *Romboutsia* (8f04c) and *Turicibacter* (62c62) were also present at higher RA in used bedding material and milk, but with no significant treatment differences. Similar findings were made for *Clostridioides difficile* (870a5), *Paeniclostridium* (0bb17), and unclassified *Oscillospiraceae* (40610, e3f70, and f1f91). *Lactobacillus*

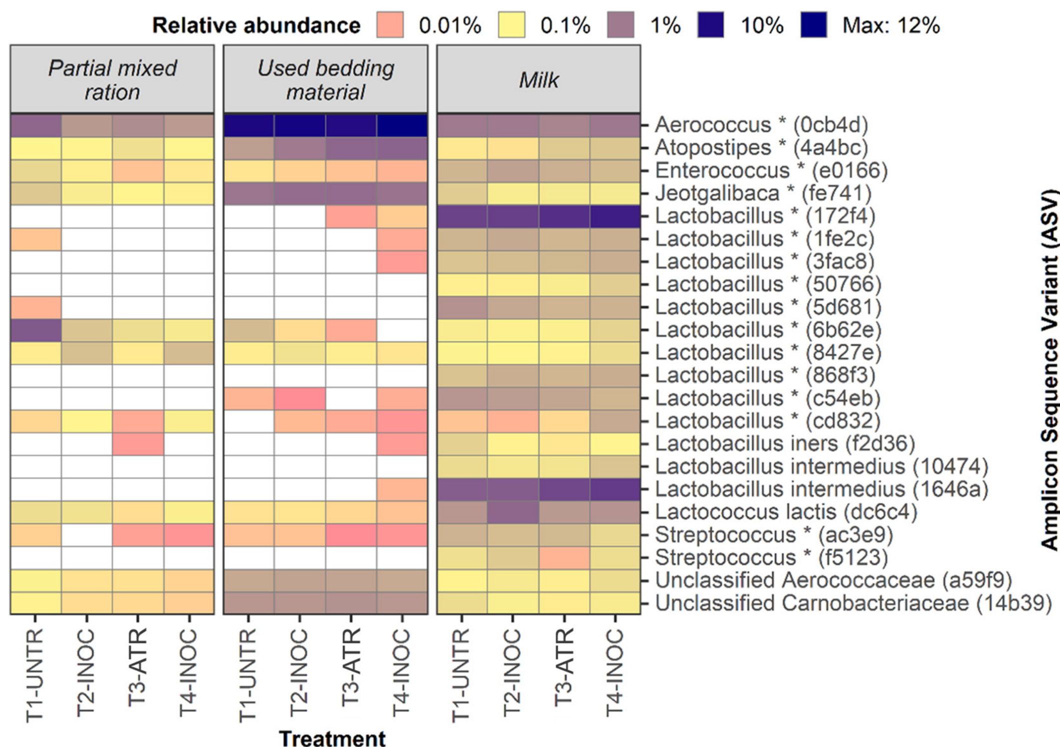

Figure 7. Heatmap showing $\log_{10}$-transformed relative abundance (RA) of *Lactobacillales* ASV found at average RA >0.1% in milk, and their concurrent RA in partial mixed ration and used bedding material during the feeding trial. The treatments were: untreated silage (T1-UNTR), inoculated silage (T2-INOC), acid-treated silage (T3-ATR), and a repeat of inoculated silage (T4-INOC). The legend scale was converted back to RA and nonpresent ASV were colored white for easier interpretation. *Indicates that the ASV was not classified further than the genus level. Max = maximum.

(172f4) and *Lactobacillus intermedius* (1646a) were the most abundant LAB in milk, with average RA of 4.6% and 2.5%, respectively. However, they were not found in the other materials and showed no significant treatment differences. Similar findings were made for *Lactococcus lactis* (dc6c4), although it was observed at low RA (>0.1%) in the PMR during all treatments except ATR. The only significant treatment difference in milk was observed for *Atopostipes* (4a4bc), which showed higher RA during the ATR and second INOC treatments compared with the first INOC. *Ralstonia* (b37c7) showed a strong tendency for higher RA in milk during UNTR, but the difference was not significant. A few more tendencies for differences between treatments were observed, but were not strong enough to overcome the variation between sampling occasions.

Further Investigation of the LAB ASV Found in Milk

Further investigation was performed by filtering out ASV belonging to the order *Lactobacillales*, resulting in a total of 716 detected LAB in the 3 materials, of which 437 were detected in milk. Of the LAB ASV found in milk, only 22 were present in average RA >0.1%, and these were summarized in a heatmap (Figure 7). Those present in highest RA were *Lactobacillus* (172f4), *Lactobacillus intermedius* (1646a), *Aerococcus* (0cb4d), and *Lactococcus lactis* (dc6c4). These 4 ASV were already included in the heatmap in Figure 6, together with the less abundant *Atopostipes* (4a4bc), *Jeotgalibaca* (fe741), *Lactobacillus* (6b62e), and unclassified *Carnobacteriaceae* (14b39).

The remaining 14 of the 22 LAB ASV in milk were not among the top 50 ASV in Figure 6, most of them (10/14) being *Lactobacillus*. Among these, only *Lactobacillus* (8427e and cd832) was clearly found in both milk and the other materials, whereas the others were in principle only found in milk, which was also the case for *Streptococcus* (ac3e9 and f5123). *Enterococcus* (e0166) was found in the PMR, but barely detected in used bedding material. Unclassified *Aerococcaceae* (a59f9) in milk was found in both used bedding material and PMR. No significant treatment differences were observed for these ASV in milk.

DISCUSSION

This study evaluated whether silages intended for dairy cows, and produced with different silage additives, affect the microbiota of the milk, and whether LAB are transferred from feed to milk. The silage treatments were each fed as PMR to 67 dairy cows for 3 wk, with one treatment fed twice to evaluate whether potential changes in milk microbiota were repeated. To our surprise, we found only minor differences in the microbiota of the different silages. The microbiota of the silages was reflected in that of the corresponding PMR, and the major bacteria in PMR were also found in used bedding material, but rarely in milk. The milk microbiota was mostly related to that of used bedding material. Abundant bacteria in milk, especially LAB, were often not found in the other materials.

Silage Additives Affected the Silages Less than Expected

In a previous study evaluating the effect of silage additives typically used in the Nordic countries on the final microbiota in laboratory-scale silages, we observed a strong effect of ensiling additives on silage microbiota, whereas herbage microbiota showed little resemblance to that of the corresponding silage (Eliasson et al., 2023). In this study, we evaluated the microbiota of silages preserved in the same way as in our previous study, but in full-scale on a farm, and evaluated whether the different silages affected the microbiota of the milk when fed to dairy cows. The core microbiota of the herbage mainly comprised *Sphingomonas*, *Xanthomonas*, and a few other non-LAB genera, whereas the silage was dominated by *Lactobacillus*, *Prevotella*, and *Pseudomonas*. The effect of silage additives on the silage microbiota was not as clear as in our previous study, although some differences between the treatments were found. At first glance, ATR tended to differ from the other treatments, with higher RA of *Lactobacillus* in the silage. Scrutiny at ASV level showed that the major silage genera comprised many different species present at varying RA. However, only a few ASV showed significant differences between the silage treatments. As in our previous study, *Lactobacillus fructivorans* was mainly associated with ATR, whereas various other LAB ASV were found in UNTR and INOC in varying proportions. Although only a few observed differences were statistically significant, we believe that the microbiota of the silages differed. With the low number of replicates per sampling week (n = 6, 2 per sampling day) and sometimes large variation between these, differences had to be major to be statistically significant.

Prevotella and Its Potential Origin

The finding of *Prevotella* in the silages was interesting, as this genus was not observed in our previous study (Eliasson et al., 2023) or in most other recent silage studies. In a laboratory-scale study by Franco et al. (2022) and the on-farm study by Kennang Ouamba et al. (2022), similar crops were ensiled, but *Prevotella* was not detected in the final silages. However, closer scrutiny of the results reported by Bayat et al. (2023) revealed a clear finding of *Prevotella* in some of their bunker silos and feed mixes. This is particularly interesting, as their study was similar to ours in many feed-related aspects. Based on the finding by Seshadri et al. (2018) that *Prevotella* is one of the dominant genera in the rumen, contamination of the barn environment by rumen bacteria is a likely explanation for the presence of *Prevotella* in feed in both our study and that by Bayat et al. (2023). Further support for this suggestion is provided by findings from Krizsan et al. (2023) of the presence of *Prevotella* at an average RA of 34.3% in rumen samples obtained from cows on the same dairy farm 2 mo before our study took place. Additionally, analysis of the raw data from the study by Ramin et al. (2023) of cow feces on the dairy farm during the period covered in the study by Krizsan et al. (2023) showed that sequences belonging to *Prevotellaceae* followed from the rumen to the feces. However, a multiple alignment with *blastn* (Zhang et al., 2000) of the most abundant *Prevotella* ASV in our study with the ASV of their studies, at its best, resulted in an alignment at 94% identity with full query cover, meaning that the sequences detected differed in at least 15 bp. This indicates that the bacteria in our study were rather distant from those found in the earlier rumen and feces samples from the same farm.

Lactobacillus Fructivorans and Acid-Treated Silage

Very few silage studies have reported *Lactobacillus fructivorans* in silages. This bacterium was detected in TMR-silage by Nishino et al. (2015), but was not discussed further until a study by Wu and Nishino (2016), who produced alfalfa silage using molasses. Interestingly, those authors found that *Lactobacillus fructivorans* did not grow well on MRS agar, but grew well on liver-infused sake agar. This could be one reason why it has not attracted much attention in previous silage studies. In both the present study and our previous ensiling study (Eliasson et al., 2023), *Lactobacillus fructivorans* was mainly found in silage made with formic and propionic acid as an additive. We found no clear connection between these acids and *Lactobacillus fructivorans* in the literature, although the bacterium is known to grow

well at high ethanol concentrations (Suzuki et al., 2008). Unfortunately, ethanol in the silages was not analyzed in this study or our previous study. However, Randby and Bakken (2021) found that silages made from crops similar to ours, with formic and propionic acid as additives, contain up to 30 g of ethanol per kilogram of DM. Following the reclassification of *Lactobacillus* into new genera (Zheng et al., 2020), *Lactobacillus fructivorans* now belongs to *Fructilactobacillus*. In the study by Bayat et al. (2023), using similar crops for ensiling, this new genus was found at the highest RA in silage made with formic and propionic acid. Thus there seems to be a rather clear connection between the bacterium and this type of silage additive, but it was not possible to evaluate the association further in this study.

Sampled Materials in the Feeding Trial Provided Different Bacterial Niches

Analyses of total bacteria count, α-diversity, and microbial composition provided complementary information that was useful in characterization of the different materials. Silage and PMR both showed low diversity and higher numbers of lactobacilli than of total bacteria, with a major part of the RA explained by *Lactobacillus*. Used bedding material showed high diversity and higher numbers of total bacteria than of lactobacilli, with a major part of the RA explained by *Acinetobacter*, *Aerococcus*, *Corynebacterium,* unclassified *Lachnospiraceae*, and unclassified *Oscillospiraceae*. Concentrate, rapeseed meal, and wood shavings also showed high diversity and higher number of total bacteria than of lactobacilli, with a major part of the RA explained by *Pantoea*, *Pedobacter*, *Pseudomonas*, *Sphingomonas*, and unclassified *Yersiniaceae*.

The exceptionally high diversity found in milk, in combination with the low total bacteria count, highlighted an important consideration when evaluating milk microbiota. The high diversity indicated that DNA from many different bacteria was present, whereas the low total bacteria count indicated that the amount of DNA representing each unique bacterium was small. This could potentially lead to bias from background contamination, as discussed by Marsh et al. (2018). Alpha diversity showed greater variation within treatment for the milk samples than for the other materials, so bias due to background contamination could have arisen in our study.

The minor difference between the microbiota in silage and PMR, despite major inclusion of concentrate and rapeseed meal in the latter, was probably due to differences in the total bacteria count and DM content between silage and PMR. On an FM basis, silage contributed almost 3 times greater volume of material, together with bacterial concentrations that were many $\log_{10}$ cfu/g higher than in concentrate and rapeseed meal. The major differences between fresh wood shavings and used bedding material were probably explained by major inclusion in bedding of e.g., animal feces with much higher bacterial load than the wood shavings. Surprisingly, among all materials analyzed, the microbiota in used bedding material showed the highest resemblance with that in milk, although the clustering of milk and used bedding material was not as tight as that for silage and PMR. The 2 clusters were also not close to each other, indicating that feed microbiota had little in common with milk microbiota.

According to Vacheyrou et al. (2011), bacteria that are useful in cheesemaking (e.g., lactobacilli and propionic acid bacteria) are frequently present on the teat surface and in the milk, but rarely in other environments in the barn (air, dust, hay). A study by Doyle et al. (2017) confirmed the contribution of teats, but also identified feces as a major contamination source of the raw milk microbiota, whereas the contribution of grass or silage was minor. Gagnon et al. (2020) found that when a novel bedding material for dairy cows was used (recycled manure solids), the raw milk microbiota changed, whereas Sun et al. (2022) observed differences in bulk milk microbiota depending on milking system and hygiene routines applied on-farm. In agreement with these studies, we found that the microbiota of silage and the corresponding PMR had little in common with that of the milk, and that the microbiota of the milk was mainly associated with that of used bedding material.

Transfer of Bacteria from Feed to Milk Was Rarely Observed

Surprisingly, *Lactobacillus acetotolerans* (e1910), *Lactobacillus fructivorans* (08c5f), *Prevotella* (f74b4), and *Pseudomonas* (15a37) were barely detected in milk. They all showed exceptionally high RA (>10%) in PMR during at least one of the treatments, and all were clearly present in used bedding material. Ouamba et al. (2023) estimated bacterial transfer at the ASV level between feed and milk to be 18% to 31%. The high RA of *Prevotella* in most materials indicated that these bacteria were well established in the barn and the surrounding environment. However, in comparison with the clear findings in both raw and pasteurized milk by Quigley et al. (2013), *Prevotella* and other core ASV in PMR were barely detectable in the milk in our study.

Aerococcus (0cb4d) showed a clear tendency to transfer from feed to milk, and was also the most abundant ASV in used bedding material. This ASV matched fully with a few species, including *Aerococcus viridans* and *Aerococcus urinaeequi*, both described in relation to mastitis (Jahan et al., 2021; Alessandri et al., 2023). The high presence of this ASV in used bedding material could

be due to its contamination by milk from cows with mastitis. Saishu et al. (2015) concluded that bedding material could be a source of *Aerococcus viridans*, based on findings from cow herds with clinical mastitis. *Acinetobacter* (8572d) also showed a clear tendency to transfer from feed to milk and was the second most abundant ASV in used bedding material. It matched fully with *Acinetobacter lwoffii* and *Prolinoborus fasciculus*, with the latter being considered an erroneous classification (Glaeser et al., 2020). Previous studies have reported clear findings of this bacterium at both the teat apex and base of the udder (Dean et al., 2021), in manure and manure lagoon (Crippen et al., 2024), and in raw milk (Guo et al., 2021). These studies, together with our findings, suggest that *Acinetobacter lwoffii* in milk mainly originates from used bedding material, contaminating the teats of the cow, but that the original source could be the feed. *Pantoea* (0951f) also showed a tendency to transfer from feed to milk, and the ASV matched fully with a few different species of *Pantoea*, mostly *Pantoea agglomerans*. This genus is mostly discussed in relation to plants (Lorenzi et al., 2022), with a few findings of *Pantoea* reported in raw milk and in pasteurized milk (Masiello et al., 2016).

CONCLUSIONS

To our surprise, we did not observe the expected effect of different ensiling treatments on silage microbiota, and we observed very limited transfer of bacteria from silage and PMR to the raw milk. *Lactobacillus* was a major genus in both feed and milk, but investigations at ASV level showed that in most cases, the ASV in these materials differed. The different materials harbored quite different microbiota, with the milk microbiota showing the highest resemblance to that of used bedding material. However, low total bacteria count in combination with high diversity indicated a risk of environmental contamination of the milk samples, and thus bias in the results. Considering that the study was conducted on a research farm rather than a commercial farm, strict hygienic measures during the feeding experiment could have contributed to the low transfer of bacteria from feed to milk.

NOTES

This work was funded by an industry PhD program of the Swedish Government (Stockholm, Sweden, reference number N2017/03895/SK), the Swedish Farmers' Foundation for Agricultural Research (Stockholm, Sweden, project R-18-26-005), the Swedish University of Agricultural Sciences (SLU.ua.2017.1.1.1-2416), and Norrmejerier Ek. Förening (Umeå, Sweden). We gratefully acknowledge the staff at Maskinringen Västerbotten (Umeå, Sweden), the dairy barn, and Norrmejerier for their work, patience and support during this major experiment. Supplemental material for this article is available at https://doi.org/10.6084/m9.figshare.26924305. All use of animals in the study and the experimental protocol were approved by the Swedish Ethics Committee on Animal Research (Permit A 6-2021), represented by the Court of Appeal for Northern Norrland in Umeå, in line with Swedish laws and regulations implementing EU Directive 2010/63/EU on animal research. Thomas Eliasson, Annika Höjer, and Karin Hallin Saedén are all employees of Norrmejerier. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: ASV = amplicon sequence variant; ATR = silage with acid treatment; FDPI = Faith's phylogenetic diversity index; FM = fresh matter; INOC = silage inoculated by starter culture; LAB = lactic acid bacteria; Max = maximum; MPCA = milk plate count agar; MRS = de Man, Rogosa, and Sharpe; NA = no colonies on plates; OG = overgrown by unknown microorganism; PC = principal coordinate; PCoA = principal coordinate analysis; RA = relative abundance; T = treatment; UNTR = silage without additive.

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