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Alpine grazing management, breed, and diet effects on coagulation properties, composition, and microbiota of dairy cow milk by commercial mountain-based herds

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ABSTRACT

Cow milk microbiota has received increased attention in recent years, not only because of its importance for human health but also because of its effect on the quality and technological properties of milk. Several studies, therefore, have investigated the effect of various production factors on the microbial composition of milk. However, most of the previous studies considered a limited number of animals from experimental or single farm, which could have biased the results. Therefore, this study aimed to understand the effect of different alpine production systems in Italy on the compositional and microbiological quality of milk, considering commercial herds with different feeding intensities and cattle breeds. The results obtained in this work indicated that the month and season of sampling (July for summer or February for winter) more than farm, breed, and cow diet exerted significant effects on cow milk parameters and microbiota. In particular, significant differences were observed for urea content in milk between sampling seasons. Differences in milk fat were mainly related to breed specific effects. From a microbiological point of view, statistically significant differences were found in presumptive lactic acid bacteria counts. Based on a culture-independent method, milk obtained in February harbored the highest number of *Firmicutes* (e.g., *Lactobacillus*) and the lowest number of *Actinobacteria* (e.g., *Corynebacterium*). Moreover, bacterial richness and diversity were higher in July during alpine pasture season indicating a significant effect of pasture feed on the growth of bacterial communities. The results of this study highlighted the effect of month or season mainly related to differences in feeding management (e.g., access to pasture during vegetation period, concentrates supplementation) on composition and microbiota in milk.

Key words: milk microbiota, mountain agriculture, cow breed, concentrate feeding, commercial herds

INTRODUCTION

Due to its role in food quality and safety, characterization of the composition and microbiota has become a focus of interest in livestock research (Addis et al., 2016; Oikonomou et al., 2020; Tilocca et al., 2020). In fact, microorganisms are responsible for milk fermentation and, through the fermentations products such as lactate, they affect a variety of technological (e.g., pH) as well as sensorial and organoleptic properties; microorganisms can also negatively affect milk quality and shelf life through the production of extracellular lipases and proteases, resulting in spoilage (Quigley et al., 2013). The raw milk microbiota can also have safety implications if there is contamination by pathogens (Quigley et al., 2013). Despite the increasing number of studies using high-throughput sequencing methods for the characterization of microbial communities of milk and dairy products (Parente et al., 2020), very little is still known about the influence of season and feeding on composition and variation of cow raw milk microbiota as many studies have been carried out in experimental farm or in single farm with few cows to exclude the farm management variable (Frétin et al., 2018; Carafa et al., 2020). For instance, Zhang et al. (2015) have shown that a high concentrate diet may lead to undesired changes in milk microbiota composition, including the increase of pathogenic and psychrotrophic bacteria associated with mastitis and poor feeding quality, respectively; however, this study included only 4 individual cows of an experimental farm. Doyle et al. (2016) have shown that grazing management, feeding, and teat preparation influenced the richness and

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composition of milk microbiota; however, they included cows from only 1 experimental herd. Moreover, Carafa et al. (2020) and Secchi et al. (2023) have investigated the influence of summer alpine grazing (transhumance) on milk microbial composition, resulting in combined effects due to changes in environment, as well as animal diet, but both studies included cows from only 1 herd. Few studies addressed the variation of milk microbiota in individual cows from commercial herds. Gagnon et al. (2020) studied the changes of the lactic acid bacteria in raw cow milk of 24 farms for type of forage used in dairy cow feeding. They did not find significant changes, but the study was limited to isolated lactic acid bacteria, without a metagenomic approach; Li et al. (2018) studied the changes of cow raw milk from 10 farms for weather conditions during a year of sampling, using a metagenomic approach. They found a higher richness in June than in December, but no information was provided about breed and feeding. Albonico et al. (2020) studied the changes of cow raw milk from 10 farms for feeding using a metagenomic approach. They found that the farm was the most significant factor affecting milk microbiota, but no information was provided about breed, and the study was limited to only 1 season of the year. There is a need to evaluate commercial farms with different production systems (feeding ratios, cattle breeds) to enable the realization of broadly based evaluations and the generation of real, practical, relevant information. Therefore, the aims of this study were to investigate coagulation properties, composition, and microbiota in raw milk samples of typical alpine herds of dairy cows on commercial South Tyrol farms and to explore the effect of breed and feeding on chemical and microbial milk traits. Furthermore, the season effect (mountain pasturing during summer and barn feeding in the winter) on milk microbiota and composition was evaluated.

This is the first time, to our knowledge, that all these factors have been considered together, using data from commercial rather than experimental herds.

MATERIALS AND METHODS

Study Design and Milk Sampling

The present study is part of the project Comparison of Dairy Farming Systems (CODA), which is running under the Action Plan for Mountain Agriculture and Food Sciences, financed by the Autonomous Province of South Tyrol (northeastern Italy). In this study, 12 South Tyrolean dairy farms were considered, rearing Brown Swiss or local dual-purpose Alpine Grey cows (Zanon et al., 2021). The farms were selected through the Breeder's Association and Dairy Association South Tyrol. The criteria for the selection of the farms were described by Flach et al. (2021). Briefly, to be considered a mountain farm, the herd must be located above 700 m above sea level, breed Brown Swiss or Alpine Grey cattle, and have a herd size of 7 to 20 dairy cows. The participation of farmers in this project was on a voluntary basis. All 12 farms were closely situated producers, located in the alpine region of South Tyrol. The longest distance between 2 producers was 67.5 km. According to the overall forage-to-concentrate feed ratio used, the 12 farms were classified in 6 low-input (**LI**) farms (forage-to-concentrate feed ratio of about 0.75:0.25 on DM basis) and 6 high-input (**HI**) farms (forage-to-concentrate feed ratio of about 0.65:0.35 on DM basis). Furthermore, the LI farms practiced summer pasturing, and dairy cows had ad libitum access to pasture during the vegetation period (June to October), whereas the HI farms in summer were fed with dry forage (sun-dried locally cut meadow grass). During the rest of the year (late October to May) cows were fed with a forage ratio constituted by a grass silage. For each cattle breed (Alpine Grey and Brown Swiss) 3 LI and 3 HI farms were available. Milk sampling was performed twice per farm, first in July 2021, when the cows were in mid lactation (100–200 DIM) and second in February 2022, when the cows were in late lactation (200–305 DIM). For each farm, 5 lactating dairy cows were selected following these required criteria: (1) absence of clinical signs of infection, (2) absence of physiological signs of subclinical infections (e.g., palpable lumps in the udders), and (3) no antibiotic treatment or anti-inflammatory medication in the 6 mo before enrollment. In addition to the milk samples, the parity was registered. Milk sampling was performed always before evening milking. Milk samples were aseptically manually collected from each animal following the recommendation for milking hygiene defined in the protocol of the Italian ClassyFarm system (ClassyFarm, 2024). Briefly, teat ends were externally cleaned with commercial pre-milking disinfectant and dried with individual towels. Pooled milk samples of the 4 glands were then collected after discarding the first streams of foremilk from each quarter. Afterward, milk samples of each of the 5 selected cows were collected in 2 sterile 50-mL tubes as homogeneous as possible among the 4 quarters. Each 50-mL milk sample tube was gently mixed by inversion. Afterward, one 50-mL tube was split into two 15-mL tubes and two 2-mL cryovials and immediately stored in a liquid nitrogen tank (−80°C). The remaining 50-mL tube was used to fill a 40-mL tube added with a chemical stabilizer for the investigation of coagulation properties, and composition. In total, 120 milk samples from 60 dairy cows, across both July and February months, were available for microbial coagulation and composition analysis.

Milk Quality Traits

Milk samples has been processed into Trentingrana Concast laboratory, the most important dairy test laboratory in the Trento province, which is accredited in conformity of the ISO IEC 17025:2017 (ISO, 2017) requirements. Milk composition traits, such as fat, protein, and lactose, were determined using a Fourier transform analyzer (**FTa**) and within the ISO 9622:2013 (ISO, 2013) accredited method. Casein and urea contents and index of milk aptitude to coagulate (**IAC**) values were also determined using FTa within laboratory internal method requirements.

Somatic cells count was determined using a flow cytometric analyzer and within ISO 13366–2:2006 (ISO, 2006) accredited method requirements. All the parameters, except IAC, has been calibrated with reference materials produced and provided by Laboratorio Standard Latte– Associazione Italiana Allevarori, which operate according and conforming to the ISO 17034:2016. The IAC was calculated according to the formula proposed by Penasa et al. (2015).

Microbiological Counts

Respective milk samples were decimally diluted in sterile peptone water and plated onto the following agar media: plate count agar (**PCA**) with skim milk (10 g/L) for the total bacterial count, in aerobic conditions for 24 h at 30°C; de Man, Rogosa and Sharpe (**MRS**) agar acidified to pH 5.5 with 5 *M* lactic acid, for cultivating mesophilic lactobacilli, incubated in anaerobic conditions (in a jar with an Anaerogen anaerobic system) for 48 h at 30°C; M17 agar, incubated in aerobic conditions for 48 h at 30 and 45°C, respectively, for cultivating mesophilic and thermophilic lactococci; and Violet Red Bile Agar (**VRBA**) for counting coliforms, in anaerobic conditions for 24 h at 37°C. All culture media and anaerobic system were purchased from Oxoid (Thermo Fisher, Waltham, MA).

Total DNA Extraction from Milk Samples and Preparation of the MiSeq Library

For total milk bacterial DNA extraction, 10 mL of milk were centrifuged at 4,000 × *g* for 1 0min at +4°C, and the supernatant was discarded. Genomic DNA was extracted from the pellet using the DNeasyPower Food Microbial Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. All DNA samples were purified by DNeasy PowerClean Pro Cleanup Kit (Qiagen, Milan, Italy) and quantified by Nanodrop8800 Fluorospectrometer (Thermo Scientific).

Amplicon library preparation, quality, and quantification of pooled libraries, and pair-end sequencing using the Illumina MiSeq system (Illumina) were performed at the Sequencing Platform, Fondazione Edmund Mach (San Michele a/Adige, Italy). Briefly, for each sample, a 464-nucleotide sequence of the V3-V4 region (Baker et al., 2003; Claesson et al., 2010) of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of the smaller amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter) according to the manufacturer's instructions; subsequently, DNA concentrations of the amplicons were determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. To ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA) using the High Sensitivity DNA Kit (Agilent). Following the quantitation, cleaned amplicons were mixed and combined in equimolar ratios.

Illumina Data Analysis and Sequences Identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights into Microbial Ecology (Qiime2, version 2020.11). Sequences were quality-filtered, trimmed, de-noized, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with multiple alignment program for amino acid or nucleotide sequences (MAFFT) and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Price et al., 2009; Katoh and Standley, 2013); Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pretrained Naive Bayes classifier based on the Greengenes 13_8 99% operational taxonomic units (**OTU**) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables. The data generated by Illumina sequencing were deposited in the National Center for Biotechnology Information Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) and are available under accession number PRJNA1003282.

Statistical Analysis

The data regarding microbiological counts were analyzed as means expressed in log cfu/mL. Statistical analysis was performed analyzing, the season, the breed and the amount of concentrated feed, as independent variables and the bacterial plate counts and milk traits as dependent variables. For bacterial counts and milk traits, statistical analysis (1-way ANOVA with post hoc Tukey honest significant difference test) was performed on the whole set of samples with 3 biological replicates (the farm) for a total of 120 milk samples without application of data transformation, as data met the assumption of normality (Shapiro-Wilk W test) and homoscedasticity (Levene test).

All the tests were performed using the STATISTICA data analysis software system, version 9.1 (StatSoft Inc. 2010; http://www.statsoft.com).

Regarding Miseq Illumina data, alpha-diversity was performed with observed OTU number and Shannon diversity index and statistical significance between groups was evaluated by Kruskal-Wallis H test in QIIME2; Beta-diversities were calculated using unweighted and weighted dissimilarity distance matrix in QIIME2. Beta-diversity distance matrix indicates differences in taxa composition between samples based on either presence-absence or quantitative species abundance data. Output matrix was ordinated using principal coordinate analysis (**PCoA**) and visualized using EMPeror (Vázquez-Baeza et al., 2013). Statistical significance of β-diversity distances between groups was assessed using PERMANOVA with 999 permutations in QIIME2.

RESULTS AND DISCUSSION

Milk Composition and Coagulation Properties

Table 1 depicts milk composition and coagulation properties of milk samples. The average parity ranged from 2.7 for HI Brow Swiss and LI Alpine Grey farms to 3.9 for HI Alpine Grey farms. Daily milk yield was higher in HI farms (27.6 kg and 24.0 kg for Brown Swiss and Alpine Grey farms, respectively) than in LI farms (21.1 kg for Brown Swiss and 17.1 kg for Alpine Grey farms, respectively). The average milk fat content was higher in farms breeding Brown Swiss (4.7% and 4.1% for HI and LI farms, respectively) than farms breeding Alpine Grey (3.9% and 3.7% for HI and LI farms, respectively). Milk protein ranged from 3.6% for LI Alpine Grey farms to 3.8% for all Brown Swiss farms. Similarly, lowest milk casein content was measured for LI Alpine Grey farms, whereas the highest was observed in HI and LI Brown Swiss farms. Lowest urea content was measured in HI Alpine Grey (16.5 mg/dL) and LI Brown Swiss (16.0 mg/dL), by converse the highest was measured in LI Alpine Grey farms (27.4 mg/dL). Finally, average SCC was generally higher in LI than in HI farms.

Effect of Farming System and Season on Chemical Composition in Milk

Results of the chemical analysis for sampling season are reported in Table 2. Milk protein and casein did not significantly ($P < 0.05$) differ among farming systems, whereas the highest milk fat content was measured in February HI Brown Swiss milk (Table 2). Generally, milk fat was higher in Brown Swiss than in Alpine Grey farms. We speculated that the lower fat amount measured in Alpine Grey cows could be due to a phenotypic peculiarity of Alpine Grey breed, whose milk is characterized by a lower fat/protein ratio, and in particular, lower fat content than other cattle breeds (Zanon et al., 2020a). Regarding SCC, LI farms showed higher values than HI farms. It is well known that higher SCC could be caused, not only by cow healthy issue but also by many environmental, management, and animal related factors (De Vliegher et al., 2018; Beaver et al., 2021). Therefore, SCC results might be due to differences in housing system, milking routine, feeding as well as pasture access. Ruegg (2017) showed in her review that hygiene man-

Table 1. Milk composition and coagulation properties of milk samples collected at the investigated farms[1]

	High input		Low input	
Item	Brown Swiss	Alpine Grey	Brown Swiss	Alpine Grey
Parity (n)	2.7 (1.6)	3.9 (3.0)	3.1 (1.9)	2.7 (1.4)
Daily milk yield (kg)	27.6 (6.1)	24.0 (6.6)	21.1 (5.0)	17.1 (6.1)
Milk fat (%)	4.7 (0.7)	3.9 (0.7)	4.1 (0.7)	3.7 (0.5)
Milk protein (%)	3.8 (0.3)	3.7 (0.4)	3.8 (0.4)	3.6 (0.4)
Milk casein (%)	3.0 (0.3)	2.9 (0.4)	3.0 (0.3)	2.8 (0.3)
Milk urea. (mg/dL)	21.1 (5.1)	16.5 (6.1)	16.0 (6.3)	27.4 (7.8)
Milk SCC (n × 1,000)	64 (54)	90 (102)	189 (163)	148 (138)
Milk IAC[2]	106 (7.1)	100 (9.3)	99 (9.3)	99 (10.2)

[1]SD are reported in the parentheses.

[2]IAC = Index of milk aptitude to coagulate.

Table 2. Effect of farming system and season on chemical composition (mean ± SD) in milk[1]

Feed	Breed	Month	Fat (g/100g)	Protein (g/100g)	Casein (g/100g)	SCC ($\times 10^3$ cell/mL)	Urea (mg/dL)	IAC
HI	Brown Swiss	July	4.4 ± 0.24^{AB}	3.8 ± 0.10^{A}	2.9 ± 0.09^{A}	65 ± 43^{B}	22.6 ± 2.3^{A}	106 ± 6.9^{A}
HI	Brown Swiss	February	4.7 ± 0.23^{A}	3.8 ± 0.09^{A}	3.0 ± 0.09^{A}	64 ± 41^{B}	19.8 ± 2.2^{A}	106 ± 7.9^{A}
HI	Alpine Grey	July	3.7 ± 0.28^{B}	3.7 ± 0.12^{A}	2.9 ± 0.12^{A}	124 ± 52^{AB}	18.2 ± 2.8^{AB}	100 ± 5.1^{A}
HI	Alpine Grey	February	3.9 ± 0.29^{B}	3.5 ± 0.13^{A}	2.8 ± 0.12^{A}	47 ± 53^{B}	13.2 ± 2.8^{B}	99 ± 12^{A}
LI[2]	Brown Swiss	July	4.2 ± 0.26^{AB}	3.8 ± 0.10^{A}	3.1 ± 0.09^{A}	195 ± 46^{A}	19.8 ± 2.2^{A}	99 ± 9.0^{A}
LI	Brown Swiss	February	4.2 ± 0.26^{AB}	3.8 ± 0.09^{A}	2.9 ± 0.10^{A}	177 ± 46^{AB}	14.9 ± 2.6^{B}	100 ± 10^{A}
LI[2]	Alpine Grey	July	3.7 ± 0.30^{B}	3.8 ± 0.13^{A}	2.9 ± 0.13^{A}	167 ± 55^{AB}	22.5 ± 2.9^{A}	98 ± 8.8^{A}
LI	Alpine Grey	February	3.7 ± 0.26^{B}	3.6 ± 0.10^{A}	2.8 ± 0.10^{A}	167 ± 47^{AB}	20.6 ± 2.6^{A}	100 ± 10^{A}

[A,B]For each column, values with different superscript letters are significantly different ($P < 0.05$, 1-way ANOVA with post hoc Tukey honest significant difference).

[1]HI = high-input farms; LI = low-input farms; IAC = Index of milk aptitude to coagulate.

[2]Ad libitum access to pasture.

agement in milking and housing is crucial for prevention of udder infections and consequently for ensuring milk quality and animal health. Milk urea content was always higher in July than in February and this difference was significant for LI Brown Swiss farms. These results are consistent with a previous study reporting that milk urea varied by stage of lactation and in late particular the milk urea concentration of cows in the late lactation was lower than in the middle lactation (Fatehi et al., 2012). Lastly, IAC was not significantly different among farming systems and sampling seasons (Table 2). This contrasts with previously published results where significant different coagulation properties were observed among cattle breeds and season of sampling (e.g., Zanon et al., 2020a,b), but is in agreement with our milk composition data in particular protein and casein amount that did not change significantly. In previous studies higher contents in proteins and casein were related to better coagulation ability (Zhang et al., 2023) and cheese yield was positively correlated with casein concentration (Jensen et al., 2012).

Milk Microbial Counts

Descriptive statistics of the microbial plate counts analysis are shown in Table 3. The coliforms counts onto VRBA were always lower than 1 log cfu/mL without significant differences. These results are in agreement with a previous study by Martin et al., (2016), where coliforms represented a minor population if compared with other bacterial groups. The low coliforms count onto VRBA indicated a good cleaning management of investigated farms as coliform count is considered an indicator of fecal and environmental contamination (Martin et al., 2023). Vice versa, considering the mesophilic microbial population, February milk showed significantly higher counts on M17–30, MRS, and PCA, than July milk samples (Table 3). These data suggest that the season of sampling could significantly influence the milk microbiota, and in particular the mesophilic fraction. By contrast the cow breed (Brown Swiss and Alpine Grey) and the use of concentrates (HI or LI) showed a limited effect on the change in the milk microbiota. These differences are likely related to the stage of lactation of the cows. Accordingly, Doyle et al. (2017) observed a significant increase of total bacterial numbers in late-lactation milk samples. This is likely explained by the fact that the microbial quality of late-lactation milk is generally poorer than that of mid-lactation milk (O'Connell et al., 2015). Moreover, the season effect is also likely due to the change in farm management; in summer (July) dairy cows from mountain farms can freely move outdoor during day, by contrast in all the same farms selected for this work, the cows were tiestall housed in winter (February). This difference in housing could affect milk microbiota; Carafa et al. (2020) found a shift in cow milk microbiota due to the free grazing in summer months. To our knowledge, there are not effective studies focused on how the housing is affecting milk microbiota. The studies interests were more focused on animal welfare and productivity than on milk microbiota when considering the housing as variable (Ouamba et al., 2022).

High-Throughput 16S rDNA Sequencing Analysis

The taxonomic composition at the phylum level is displayed in Table 4 and a taxa bacterial composition of the milk is represented in Figure 1. Clear differences in the bacterial profiles between the July and February milk samples were observed.

Six different taxa, *Corynebacterium, Staphylococcus, Aerococcaceae, Lactobacillus, Leuconostoc*, and *Clostridia* taxa (*Clostridia* taxa was mainly constituted by *Lachnospiraceae, Peptostreptococcaceae,* and *Ruminococcaceae*) were predominantly found. *Staphylococcus, Aerococcaceae,* and *Clostridia* taxa (*Lachnospiraceae, Peptostreptococcaceae,* and *Ruminococcaceae*) were part of the dominant microbiota in all the milk samples;

Table 3. Microbial counts (mean ± SD) of raw milk samples collected from farms clustered in 6 groups of 3 farms each; n = 15 cows, sampled in July and February[1]

Feed	Breed	Month	PCA	MRS	M17–30	M17–45	VRBA-T
HI	Brown Swiss	July	2.5 ± 0.84^{A}	0.1 ± 0.47^{A}	0.5 ± 0.97^{A}	$<10^{A}$	$<10^{A}$
HI	Brown Swiss	February	3.6 ± 0.93^{BC}	1.2 ± 1.5^{B}	1.7 ± 1.6^{A}	1.7 ± 1.5^{B}	0.2 ± 0.3^{A}
HI	Alpine Grey	July	3.3 ± 0.73^{B}	1.5 ± 1.5^{BC}	1.7 ± 1.8^{A}	1.4 ± 1.6^{AB}	0.1 ± 0.28^{A}
HI	Alpine Grey	February	4.1 ± 1.2^{C}	1.6 ± 1.5^{BC}	3.2 ± 1.0^{BC}	1.5 ± 1.6^{B}	0.2 ± 0.31^{A}
LI[2]	Brown Swiss	July	1.9 ± 1.9^{A}	1.1 ± 0.78^{B}	1.3 ± 1.1^{A}	0.5 ± 1.1^{AB}	0.7 ± 0.00^{A}
LI	Brown Swiss	February	4.4 ± 0.98^{C}	1.9 ± 1.9^{BC}	3.8 ± 1.2^{C}	1.6 ± 1.8^{B}	0.6 ± 1.3^{A}
LI[2]	Alpine Grey	July	3.2 ± 0.99^{B}	1.9 ± 1.1^{BC}	2.6 ± 1.5^{B}	1.2 ± 1.3^{AB}	0.7 ± 0.99^{A}
LI	Alpine Grey	February	4.6 ± 1.2^{C}	2.3 ± 1.5^{C}	4.2 ± 1.3^{C}	1.0 ± 1.5^{AB}	0.9 ± 1.5^{A}

[A–C]For each column, bacterial count values with A, B, and C superscripts are significantly different ($P < 0.05$, 1-way ANOVA with post hoc Tukey honest significant difference).

[1]Each group was different according to the amount of concentrate used in the feed ratio on DM basis, low-input farms (LI: forage-to-concentrate feed ratio of about 0.75:0.25) and high-input farms (HI: forage-to-concentrate feed ratio of about 0.65:0.35); and according to the breed (Brown Swiss and Alpine Grey). PCA: total mesophilic aerobic bacteria; MRS: total mesophilic lactobacilli; M17–45: total thermophilic lactococci; M17–30: total mesophilic lactococci; VRBA-T: total coliform as determined on VRBA; <1 = no colony was grown on the plate related to the first dilution.

[2]Ad libitum access to pasture.

Lactobacillus and *Leuconostoc* were most abundant in February milk (relative abundance mean always over 25% in February milk samples, Figure 1). *Corynebacterium* genus was a constant presence in all the samples (relative abundance mean in the range of 1%–15%) except for LI Alpine Grey milk sampled in February where *Corynebacterium* was not found. *Staphylococcus* (4%–35% relative abundance in all milk samples) is the most frequently isolated genus of bacteria on the cow teat skin (Braem et al., 2014) and together with *Aerococcaceae* (1%–10% relative abundance in all milk samples, with the exception of HI Alpine Grey milk sampled in February where *Aerococcaceae* relative abundance was about 15%) was found as part of the dominant microbiota in all milk samples without significant differences for feed, concentration or breed. These results are in agree with Sun et al., (2021) and Yap et al., (2024) that found these 2 taxa present as dominant in at least the 90% of

Table 4. Phylum composition (expressed as percent) in LI and HI farms milk samples as revealed by high-throughput sequencing analysis

	Brown Swiss		Alpine Grey		Brown Swiss		Alpine Grey	
	HI		HI		LI		LI	
Item	July	Feb	July	Feb	July	Feb	July	Feb
Archea	0.077	0.000	0.250	0.000	0.104	0.000	0.175	0.000
Acidobacteria	0.023	0.000	0.042	0.000	0.207	0.000	0.402	0.000
Actinobacteria	17.150	6.279	6.446	5.090	19.753	1.634	11.773	1.291
Armatimonadetes	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.000
Bacteroidetes	10.886	2.515	9.113	0.216	9.773	4.496	13.430	1.759
Chloroflexi	0.985	0.000	0.000	0.000	0.178	0.032	0.445	0.000
Cyanobacteria	1.991	0.946	3.544	0.380	6.690	0.092	8.409	0.696
Elusimicrobia	0.011	0.000	0.000	0.000	0.006	0.000	0.016	0.000
Fibrobacteres	0.077	0.000	0.000	0.000	0.000	0.000	0.040	0.000
Firmicutes	56.390	85.920	71.365	93.496	52.876	88.730	50.685	94.762
Fusobacteria	0.011	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Gemmatimonadetes	0.117	0.000	0.000	0.000	0.062	0.000	0.056	0.000
Lentisphaerae	0.021	0.000	0.026	0.009	0.029	0.036	0.041	0.000
Nitrospirae	0.004	0.000	0.000	0.000	0.016	0.000	0.028	0.000
Planctomycetes	0.436	0.085	0.079	0.000	0.121	0.071	0.325	0.000
Proteobacteria	10.503	4.192	7.635	0.809	8.772	4.816	12.526	1.366
Spirochaetes	0.097	0.014	0.104	0.000	0.085	0.000	0.270	0.126
Synergistetes	0.057	0.000	0.000	0.000	0.006	0.000	0.000	0.000
TM7	0.338	0.048	0.329	0.000	0.637	0.035	0.346	0.000
Tenericutes	0.086	0.000	0.126	0.000	0.100	0.000	0.114	0.000
Verrucomicrobia	0.550	0.000	0.791	0.000	0.496	0.000	0.836	0.000
[Thermi]	0.208	0.000	0.026	0.000	0.028	0.000	0.016	0.000

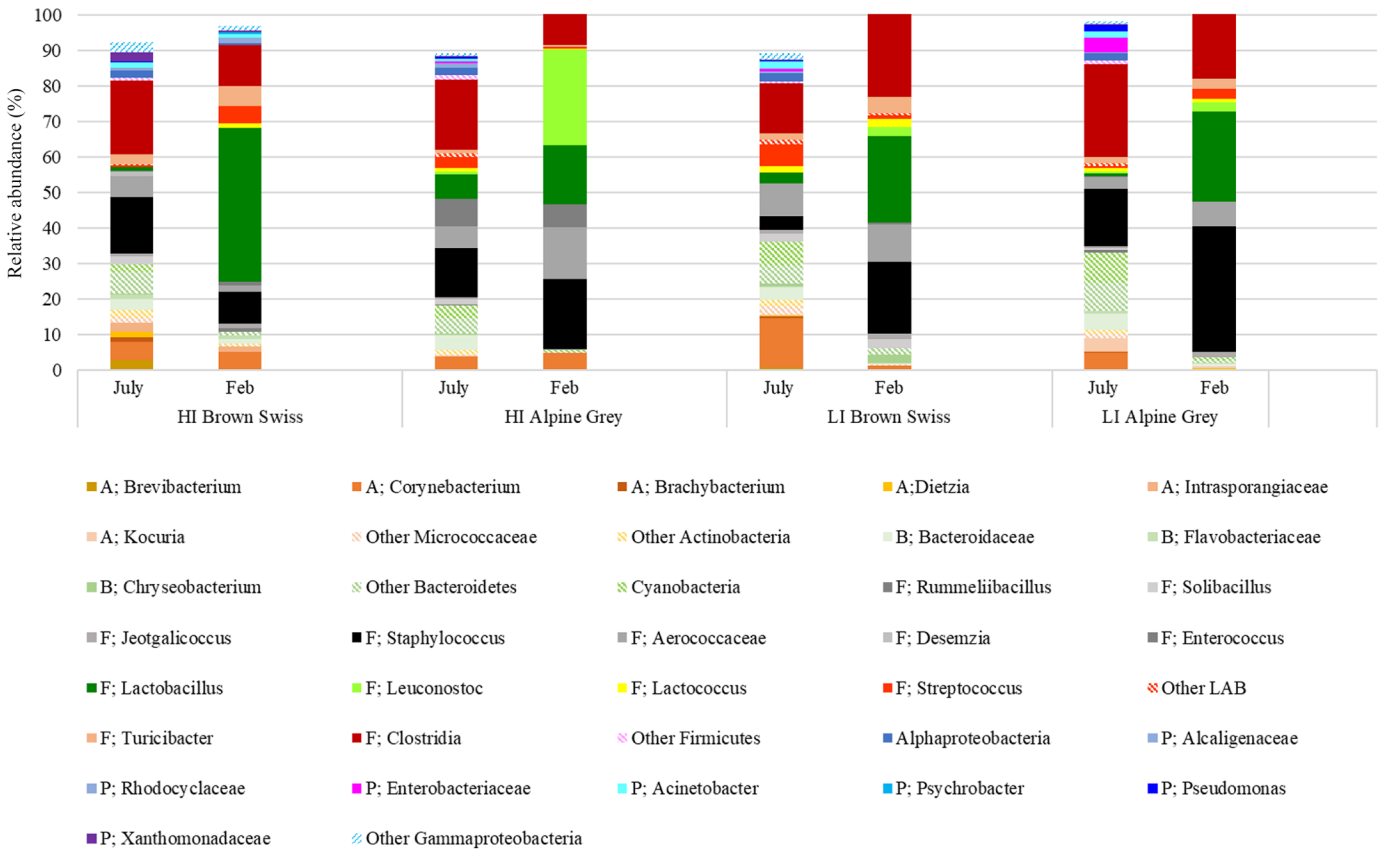

Figure 1. Relative abundances (%) of bacterial taxa identified by MiSeq Illumina in raw milk samples collected from 5 individual cows for each farm in July and February. LI = low-input farms; HI = high-input farms; A = *Actinobacteria*; B = *Bacteroidetes*; F = *Firmicutes*; and P = *Proteobacteria*.

bulk tank milk samples. The sequences belonging to *Clostridia* (*Lachnospiraceae, Peptostreptococcaceae,* and *Ruminococcaceae*) were also detected in all milk samples; both *Aerococcaceae* and *Clostridia* could be environmental milk contaminants bacterial taxa because they were already found as the most abundant in the dairy farm environment (Doyle et al., 2017; Falardeau et al., 2019); in particular, *Clostridia* in raw cow milk has been correlated with grass or maize silage (Raats et al., 2011). Also considering the phylum composition (Table 4) *Actinobacteria, Bacteroidetes*, and *Proteobacteria* were found in higher relative abundance in July milk samples, in agree with Metzger et al. (2018) that found a strong effect of season of the year and of stage of lactation, which resulted in increasing richness from winter to summer and in significant changes in the relative abundance of milk OTU including *Corynebacterium* and *Bacteroidetes*. They attributed the *Bacteroidetes* increase in July to contamination from sand bedding or the combination of bedding material and physiological changes during lactation. Interestingly, milk from quarter with chronic inflammation showed the greatest seasonal changes.

To evaluate differences between the bacterial microbiota of LI and HI milk, sampled in different months and from different breeds, comparative analyses were performed with the sequences generated in this study. The microbial richness (Observed OTUs number) and diversity (Shannon index) were compared between the LI and HI milk from different months and cows (Table 5). Based

Table 5. Richness expressed as observed OTU number (Obs OTU) and diversity expressed by Shannon index of the bacterial communities identified by 16S amplicon sequencing of the low input (LI) and high input (HI) milk sampled in July and February[1]

Feed	Breed	Month	Obs OTU	Shannon index
HI	Brown Swiss	July	235 ± 54^{A}	0.728 ± 0.069^{A}
HI	Brown Swiss	February	44 ± 16^{B}	0.480 ± 0.045^{B}
HI	Alpine Grey	July	193 ± 67^{A}	0.682 ± 0.093^{A}
HI	Alpine Grey	February	30 ± 9^{B}	0.442 ± 0.056^{B}
LI	Brown Swiss	July	204 ± 49^{A}	0.702 ± 0.052^{A}
LI	Brown Swiss	February	41 ± 24^{B}	0.457 ± 0.101^{B}
LI	Alpine Grey	July	198 ± 68^{A}	0.655 ± 0.132^{A}
LI	Alpine Grey	February	27 ± 16^{B}	0.405 ± 0.211^{B}

[A,B]Different superscript letters within breed with different production groups (HI or LI) are significantly different ($P < 0.05$).

[1]Results are shown as mean values ± SD of 15 values (15 cows).

on both the observed OTU number and Shannon index, the July milk contained a higher level of bacterial richness and diversity than February milk samples (P-value was always <0.01). In contrast, no significant difference in α diversity was observed between milk samples when they were grouped by feed (P = 0.579 and 0.424 for observed OTU number and Shannon index, respectively), farm (P = 0.951, and 0.858 for observed OTU number and Shannon index, respectively) or breed (P = 0.939, and 0.697 for observed OTU number and Shannon index, respectively). These results agree with previous works by Carafa et al. (2020) confirming that the season and stage of lactation had a high and significant effect on the milk microbial richness and diversity better than farm, breed, feeding, and with Yap et al., (2024) that found summer milk significantly richer than winter milk.

Two distance matrixes were created based on weighted and unweighted Unifrac indexes and were used to calculate distances between pairs of samples, representing how closely related samples are. The PCoA based on unweighted (Figure 2A) and weighted (Figure 2B) Unifrac distance matrixes showed similar results. The combination of the first 2 axes explained 32.07 and 58.13 of the variances based on unweighted and weighted distance matrix, respectively. Samples were colored according to the month of sampling and shaped according to the feeding to visualize and identify samples. Samples closer to one another are more similar than those that are further away from each other. There was a sample clustering observed for July and February milk in both PCoA. Looking at the visualization of the weighted Unifrac distance matrix (Figure 2B), the February samples were more closely grouped, whereas the July samples were spread more widely across the PCoA space. Because the unweighted Unifrac distance accounts for the presence or absence of OTU, whereas the weighted Unifrac for abundance too, the similar results of these analyses suggest that both microbial composition and individual microbial abundance drive the distance between July and February samples. No pattern was observed when samples were colored according to the individual breed, nor to the farm of sampling (data not shown). The PERMANOVA analysis revealed high (Pseudo-F) and significant microbial composition differences between July and February milk samples (unweighted Unifrac pseudo-F = 26.31, P = 0.001; weighted Unifrac pseudo-F = 22.04, P = 0.001), confirming again that the season and lactation stage had a high and significant effect on the milk microbial community. By contrast, no significance was observed when samples were grouped according to the breed (unweighted Unifrac P = 0.104; weighted Unifrac P = 0.391) nor to the feed (unweighted Unifrac P = 0.117) and nor to the farm (unweighted Unifrac P = 0.404). Considering the weighted Unifrac PERMANOVA analysis, a significant difference was observed for the feed (weighted Unifrac pseudo-F = 2.34, P = 0.045). This could mean that the abundance more than the species could be slightly affected by a change in the diet. In fact, looking at the graph in Figure 1, we could notice that with a HI diet, there is an increase of *Enterococcus, Lactobacillus* and *Leuconostoc* genera in milk and a general decrease of *Clostridia* and *Enterobacteriaceae* comparing with LI diet. We speculated that these taxa could come from a cross-contamination between cow feed and milk because these taxa were found to be higher in HI milk samples and in February when no free grazing is allowed to the

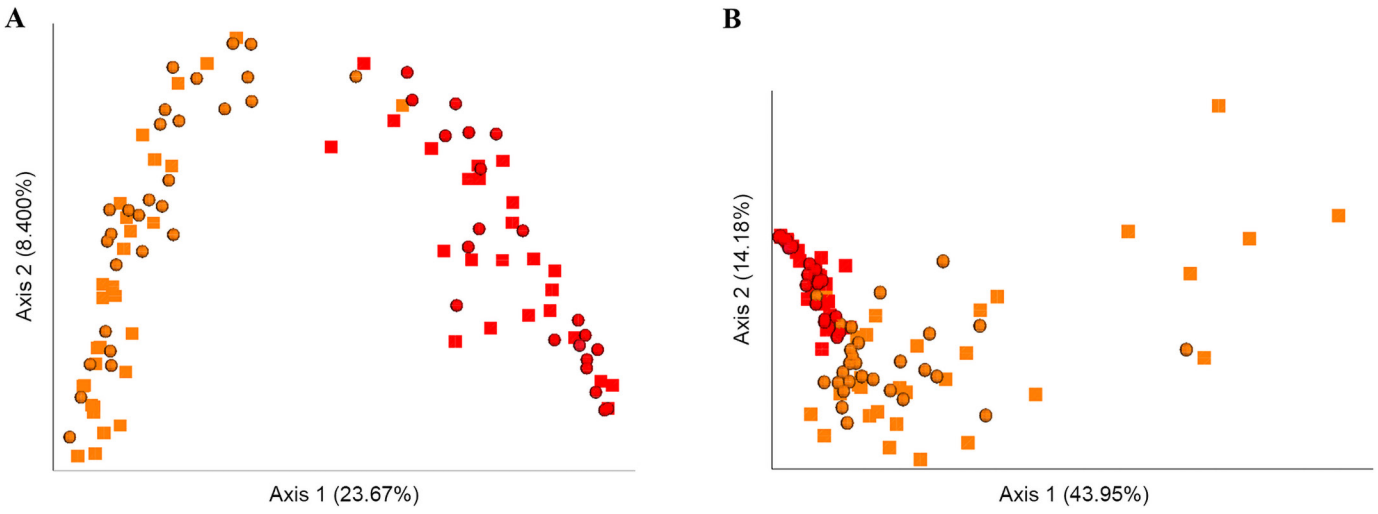

Figure 2. Beta-diversity microbiota changes based on feeding and month of sampling. Two principal coordinate analysis ordinations using unweighted (A) and weighted (B) Unifrac distances were performed to visualize microbial community OTU differences across the 2 feeding systems (HI = circles; LI = squares) and month of sampling (red = February; orange = July).

cows. In a previous work by Du et al. (2020), *Lactobacillaceae* and *Leuconostocaceae* were the most abundant taxa found in feed constituting at least 40% of feed microbiota. *Lactobacillus* is a beneficial, health-promoting species for cows and is associated with healthy udders compared with mastitic udders (Ma et al., 2016). In fact, *Lactobacillus* was used together with *Bifidobacterium* to effectively treat mastitis, particularly through oral delivery (Rainard and Foucras, 2018). In contrast, Zhang et al. (2015) observed a higher percentage of mastitis causing pathogens when feeding high concentrate diets with 70% concentrates. Importantly too, *Lactobacillus* and *Leuconostoc* are desired bacteria for the preservation of the dairy productions (Moula Ali et al., 2022), so their resulting higher abundance in the HI diet could have a positive effect on the use of milk for dairy productions purposes.

CONCLUSIONS

In the present study chemical and microbial composition as well as coagulation properties of milk from Alpine dairy farming systems with different feeding ratios, husbandry system and cattle breeds were investigated. Hereby, for the first time, a larger number of commercial farms than in previous studies was considered to achieve a broad-based evaluation and to generate practical relevant information. Results revealed that month of sampling had a notable effect on milk urea content as well as microbial composition. Furthermore, the higher energy content in feed in the HI diet favored the growth of beneficial bacteria for udder health as well as for product quality. Moreover, bacterial richness and diversity was higher in July, indicating a significant effect of pasture feed on the growth of bacterial communities. Future research should consider lactation stage and season when characterizing the relation between diet and milk microbiota as well as further investigate the effect of different feed concentrate supplementations on milk microbiota.

NOTES

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Nonstandard abbreviations used: FTa = Fourier transform analyzer; HI = high input; IAC = Index of milk Aptitude to Coagulate; LI = low input; MRS = de Man, Rogosa and Sharpe; OTU = operational taxonomic units; Obs OTU = observed OTU; PCA = plate count agar; PCoA = principal coordinate analysis; VRBA = Violet Red Bile Agar; VRBA-T = total coliform as determined on VRBA.

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