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Aloe arborescens supplementation in drying-off dairy cows: influence on rumen, rectum and milk microbiomes

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Abstract

Background In the context of the RABOLA project, which aimed to identify operational practices that lead to the reduction of antibiotic use in dairy cattle farming, lyophilised *Aloe arborescens* was administered orally to cows during the dry-off and peripartum periods. In this specific paper we wanted to examine whether oral administration of *Aloe arborescens*, in combination with the topical application of a teat sealant could exert an effect on the microbial populations of three cow microbiomes (rumen, milk, rectum), between dry-off and peripartum. Dry-off and peripartum are critical physiological phases of the cow's life, where both the mammary gland and the gastrointestinal tract undergo dramatic modifications, hence the relevance of evaluating the effects of dietary treatments.

Methods Thirty multiparous dairy cows were randomly allocated to three groups: Control (antibiotic treatment and internal teat sealant), Sealant (only internal teat sealant) and Aloe (internal teat sealant and *Aloe arborescens* homogenate administered orally). For 16S rRNA gene sequencing, rumen, rectum and milk samples were collected, not synchronously, at the most critical timepoints around dry-off and calving, considering the physiological activity of each biological site.

Results The rumen microbiome was predominantly characterized by Bacteroidetes and Firmicutes followed by Proteobacteria, while the rectum exhibited a prevalence of Firmicutes and Bacteroidetes. The milk microbiome mainly comprised Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes. Alistipes spp., Ruminococcaceae UCG-10 group, Prevotellaceae UCG-001 group, and Bacteroides spp., involved in cellulose and hemicellulose degradation, enhancement of energy metabolism, and peptide breakdown, showed increment in the rectum microbiome with Aloe supplementation. The rectum microbiome in the Aloe group exhibited a significant increase in the Firmicutes to Bacteroidetes ratio and alpha-diversity at seven days after dry-off period. Beta-diversity showed a significant separation between treatments for the rectum and milk microbiomes. Aloe supplementation seemed to enrich milk microbial composition, whereas the Sealant group showed greater diversity compared to the Control group, albeit this included an increase in microorganisms frequently associated with mastitis.

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Conclusions *Aloe arborescens* administration during the dry-off period did not demonstrate any observable impact on the microbial composition of the rumen, a finding further supported by volatilome analysis. Instead, the oral Aloe supplementation at dry-off appears to significantly influence the composition of the dairy cow rectum and milk microbiomes in the following lactation.

Keywords *Aloe arborescens*, Dry cow period, Microbiome, Milk, Rumen, Rectum

Interpretive summary
This study aimed to investigate the effects of supplementation with polysaccharide-rich *Aloe arborescens*, which has anti-inflammatory, immunostimulant, antibacterial, and antioxidant properties, on the rumen, rectum and milk microbiomes of dairy cows during the transition period. This dietary supplementation appears to exert a significant influence on the composition of the rectum and milk microbiomes in dairy cows, modulating both richness and microbial composition, but it has no effect at the rumen level.

Background

Intra-mammary infusion with long-acting antibiotics at dry-off (dry cow therapy, DCT) is a long-standing practice in the dairy industry [1] to (i) cure existing infections at dry-off, (ii) decrease the risk of new infections during the dry period, (iii) reduce the risk of clinical mastitis at the start of next lactation, and (iv) decrease the somatic cell count during the early lactation. For a long time, DCT has been used to treat all quarters of all cows with antibiotic administration (blanket DCT, or BDCT); however, more recently the approach of selectively treating only infected cows or quarters (selective DCT, or SDCT) has emerged [2]. Over the years, the problem of antibiotic resistance in veterinary and human medicine has become progressively more alarming, with an increased hazard of ineffective treatment of bacterial infections [3]. Consequently, it is apparent that the unnecessary administration of antibiotics is clearly unsustainable, and therefore it becomes crucial to be able to control direct antibiotic use and the release of antibiotic residues in the environment [4, 5]. In several European countries there has been a drastic reduction in the use of antibiotics in livestock, with the approval of a new legislation (EU regulation 2019/6) which bans the use of antibiotics for preventive purposes; the antimicrobial therapy can no longer be applied to all animals in a preventive manner (as in BDCT), but can only be administered for treatment of diagnosed intra-mammary infection (IMI) based on somatic cell count (SCC) or bacterial culture, as in SDCT [3].

From the farm management perspective, around parturition cows have difficulties to adapt to the nutrient needs for lactation [6], and this results in a physiological imbalance with incremented risk of digestive, metabolic and infectious diseases [7–9]. The use of plant-based additives with proven nutraceutical properties has emerged as a promising option to improve animal health, especially during critical phases like peripartum and the drying period. Nutraceuticals, including dietary supplements, herbal products, and processed feeds, have demonstrated potential in supporting the modulation of rumen fermentation and gut microbiota as well as exerting effects on the immune system and metabolic activities. These effects are primarily attributed to their antioxidant, antimicrobial, and immune-stimulating and /or modulating properties, particularly in the period before and after parturition [6], when inflammatory phenomena are frequent and the acute phase response in the liver is particularly severe [8].

Given its therapeutic properties *Aloe* spp. has been widely used in traditional medicine for years: out of over 400 *Aloe* species belonging to the family Liliaceae, the most therapeutically relevant are *Aloe vera*, *Aloe arborescens* and *Aloe ferox* [10]. These species have high water content ($\sim$ 99%) and the remaining 0.5-1.0% of solid material is reported to contain more than 75 different potentially active compounds, including water- and fat-soluble vitamins, minerals, enzymes, simple and complex polysaccharides, phenolic compounds and organic acids [11]. Many of these compounds reveal beneficial effects with anti-inflammatory, immune-stimulant, anti-hyperlipidemic, anti-bacterial and antioxidant properties [12].

Further yet, in animals (as well as in humans) the microbiome of the gastrointestinal tract is involved in maintaining the health homeostatis of individuals, with a principal role in the host's barrier defence mechanism and in numerous metabolic, physiological, nutritional and immunological processes [13]. In ruminants, the physiological role of gastrointestinal microbiomes is especially complex, given the presence of the rumen which acts as the major gut microbiome. Among all the environmental factors, diet and dietary supplementations are known to have a great impact on the composition and metabolic activity of the gut microbiome [14].

Considering all of the above, it is therefore of scientific and practical interest to evaluate the effects of *Aloe* supplementation, in addition to teat sealant, on the, microbial populations of the rumen, rectum and mammary gland of cows treated and not with antibiotics at dry off and around calving.

Cattaneo et al. [15] evaluated the effect on the rumen activity, immunometabolic profile and milk yield of a dietary supplementation with lyophilized *Aloe arborescens* in cows that did not receive antibiotic therapy the week before and after dry-off. Using the same animals and the same experimental design, the aim of the present study was to evaluate the influence of lyophilized *Aloe arborescens* dietary supplementation on the microbial populations of the rumen, rectum and milk of dairy cows treated with: (i) intramammary antibiotics (BDCT), (ii) teat sealant, and (iii) teat sealant plus oral administration of lyophilized *Aloe.* The three microbiomes were sampled at most critical timepoints: before, at and after dry-off, and after calving (milk microbiome only), based on the physiological activity of each biological site. For this reason, sampling was not synchronous. The results presented here offer a comprehensive overview of the effect on multiple cow microbiomes when moving from BDCT to SDCT replacing the prophylactic use of antimicrobials with teat sealant and aloe administration.

Materials and methods
Experimental design and sampling
In the context of the RABOLA project, a large experiment was carried out, which involved 30 multiparous dairy cows randomly allocated to three groups of 10 healthy cows each: (1) control group (Control) – cows dried off following the typical antibiotic treatment (Mamyzin, Boehringer Ing.Anim.H., Italy) and the application of internal teat sealant (Noroseal, Norbrook Laboratories Limited); (2) cows dried off using only the internal teat sealant (Sealant); (3) cows with the internal teat sealant and oral administration of 200 mL per day of whole leaf homogenate of *Aloe arborescens* supplemented to their diet, from −7 to +7 days from the dry off (Aloe). The dose of *Aloe* was calculated so to provide similar dry matter amount to all animals [15].

Full details on cow enrolment and diet, and on the *Aloe arborescens* supplementation are reported in Cattaneo et al. [15]. Briefly, only healthy cows, without intramammary infections due to major pathogens, were recruited: animal's groups were balanced for parity, previous lactation length, and somatic cell counts (SCC) record. Rations were formulated according to the National Research Council [16] guidelines. For the microbiome analysis, rumen samples were collected at -14 (T1 in this study) and 0 (T2 in this study) days from dry-off; rectum samples were collected at -14 (T1), 0 (T2) and 7 (T3)

days from dry-off, while milk samples at -14 (T1) days from dry-off and 28 (T4) days after the following calving. Rumen fluid was collected before feeding using a rumen probe (Ruminator; profs-products.com). The first liter of rumen fluid was discarded to avoid saliva contamination, and the next 0.5 L was retained for sampling. Clean jar glass was used for each cow and the probe was flushed thoroughly with tap water between cows. Fecal samples were collected from spontaneous defecation in sterile plastic jars. Milk samples were collected in sterile 15 mL vial after cleaning the operator's nitrile gloves and cow teats with alcohol and unloading the first five shots. Sampling was not synchronous due to the different physiological activities of each biological site. The study design with the detailed sampling collection scheme is reported in Supplementary Material (Supplementary File - Fig. 1), where the sampling timepoints are highlighted (circled).

Microbiological analysis
One-milliliter raw milk samples (T1, T4) were serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology, Barcelona, Spain) and inoculated in different media for bacterial count. Total mesophilic and psychrophilic bacteria (TMB) were counted on Petrifilm Aerobic Count Plate (3 M, Minneapolis, MN, USA) at 30° C for 72 h and 7 °C for 10 days, respectively; Enterobacteriaceae and *Escherichia coli* were detected on SENECA agar (Biolife Italiana, Milan, Italy) at 37 °C for 24 h while coagulase-positive *Staphylococcus* were counted on Baird Parker agar with a rabbit plasma fibrinogen supplement (BP+RPF) (Biolife Italiana) incubated at 37 °C for 24–48 h. Lactic acid bacteria (LAB) were enumerated on de Man Rogosa Sharpe (MRS) agar ((Scharlau Microbiology) under anaerobic conditions (AnaerocultA, Merck, Darmstad, Germany) at 37 °C for 72 h while the content of enterococci was determined in Kanamycin Aesculin Azide (KAA) agar (Biolife Italiana) at 37 °C for 48 h.

Fecal samples (10 g) (T1, T2 and T3) were homogenized in 90 mL of a 2% (w/v) sterile Buffered Peptone water (Biolife Italiana) for 2 min in a Stomacher Bag-Mixer (Interscience, St. Nom, France). Samples were serially diluted in quarter-strength Ringer's solution (Scharlau Microbiology) and plated in the same media used for the analysis of raw milk samples.

DNA extraction and purification
Rumen liquor samples – Deoxyribonucleic acid extraction was performed from 0.25 g of lyophilized rumen fluid samples following the protocol described in literature [17]. This protocol is widely used to extract bacterial DNA from rumen samples combining chemical and mechanical sample's lysis [18].

Rectum samples - Using the commercial QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany), the
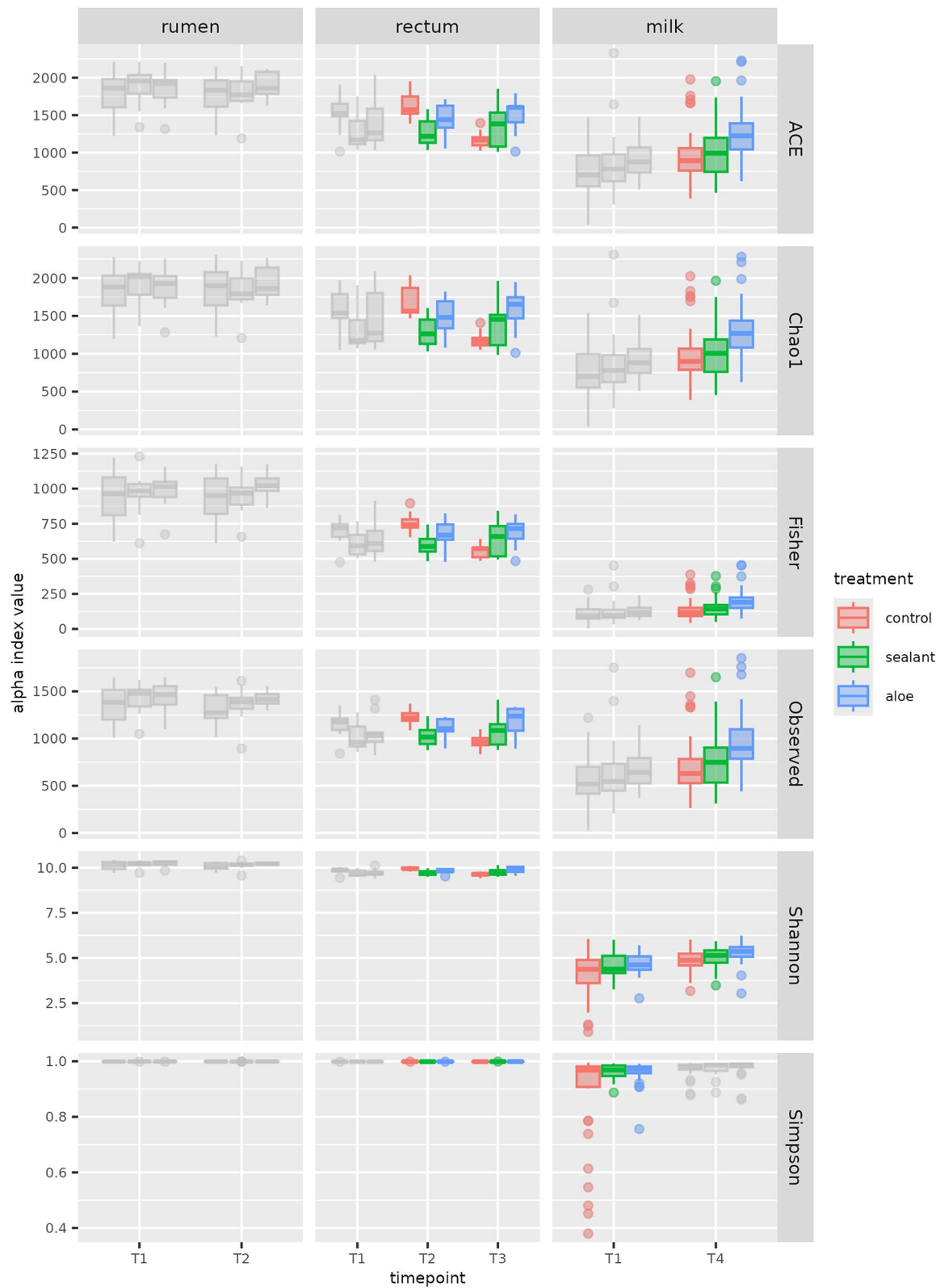
Fig. 1 Distribution of alpha-diversity. Boxplots of the alpha-diversity indexes (ACE, Chao1, Fisher's alpha, observed n. of OTUs, Shannon and Simpson diversity) in the three experimental groups (Control, Sealant, Aloe) at the different timepoints (x-axis) for the three microbiomes (rumen, rectum and milk). The significance of differences in alpha-diversity indexes between experimental groups for the rumen (left), rectum (center), and milk microbiomes (right) at different time points was highlighted

bacterial DNA was extracted from each rectum sample following the manufacturer's protocol.

Milk samples - For each quarter, 5 mL of milk were analysed by using a DNA extraction method based on the combination of a chaotropic agent, guanidium thiocyanate, with silica particles, to obtain bacterial cell lysis and nuclease inactivation as previously described [19, 20].

All the DNA samples were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to check the quality and the quantity. The isolated DNA was stored at −20 ℃ until use.

16S rRNA gene library preparation and sequencing

Bacterial DNA was amplified using the primers described in literature [21] which target the V3–V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25 µL volume per sample.

For rumen liquor and rectum samples, a total of 12.5 µL of KAPA HIFI Master Mix 2× (Kapa Biosystems, Inc., Wilmington, MA, USA) and 0.2 µL of each primer (100 µM) were added to 2 µL of genomic DNA (5 ng/µL). Blank controls (i.e., no DNA template added to the reaction) and positive sample (DNA extracted from the same type of samples and previously analysed) were also performed. For DNA from milk samples, a total of 12.5 µL of Phusion High-Fidelity Master Mix 2x (ThermoFisher Scientific, Waltham, MA, USA) and 0.2 µL of each primer (100 µM) were added to 2 µL of genomic DNA (5 ng/µL).

A first amplification step was performed in an Applied Biosystem 2,700 thermal cycler (ThermoFisher Scientific). For rumen and rectum, the samples were denatured at 95 ℃ for 5 min, followed by 25 cycles with a denaturing step at 95 ℃ for 1 min, annealing at 56 ℃ for 1 min and extension at 72 ℃ for 1 min, with a final extension at 72 ℃ for 7 min. For milk, samples were denatured at 98 ℃ for 3 min, followed by 25 cycles with a denaturing step at 98 ℃ for 30 s, annealing at 56 ℃ for 1 min and extension at 72 ℃ for 1 min, with a final extension at 72 ℃ for 7 min.

Amplicons were cleaned with Agencourt AMPure XP (Beckman, Coulter Brea, CA, USA) and libraries were prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). The libraries obtained were quantified by Real Time PCR with KAPA Library Quantification Kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportion and sequenced in three different MiSeq (Illumina) runs with 2×250-base paired-end reads each.

Volatilome analysis on rumen samples

The volatilome produced by bacteria in rumen liquor samples was determined by means of Head-Space Solid Phase Micro Extraction module on 30 multiparous dry cows divided in three different groups, with 10 cows for each group, as described in experimental design, and analysed at timepoint T1 (before treatment) and timepoint T2 (at dry-off) in each group of cows. An aliquot of 2.5 g of rumen liquor was weighed in a 20 ml head-space glass bottle, added 3 g of sodium chloride, to emphasize the passage of volatile molecules into the headspace, sealed with a PTFE-silicone septum and analysed by means of a Combi-Pal automated sampler (CTC Analytics AG, Zwingen, Switzerland) coupled to an Agilent 6890 gas chromatograph with an Agilent 5975 mass spectrometric detector (Agilent Technologies) and a polar column [22]. The chromatographic conditions were those present in literature [23] and the volatile compounds were identified using the Wiley 7n-1 MS library on Agilent MSD ChemStation® software (Agilent Technologies Inc.) and calculated by the peak area (arbitrary units) of the corresponding selected ion.

Milk fatty acids analysis

Milk fatty acid (FA) profile was determined in milk of the cows before (T1) and after (T4) administration of *Aloe arborescens*. The milk fat extraction from the samples was obtained by means of centrifugation of the milk for 20 min at 4100 g at 4 ℃. The obtained cream was centrifuged for 20 min at 15,060 g in a room temperature centrifuge (Eppendorf-centrifuge 5425) [24] and the clear fat obtained used for base-catalysed transesterification of the FA according to Christie [25]. The FA methyl esters in hexane were injected into a gas chromatograph (GC) Agilent 7890 GC system (Agilent Technologies) equipped with on-column injector, and an FID detector. The separation was performed on a 100% dimethylpolysiloxane column (CP-Sil88 for FAME, 100 m×0.25 mm×20 µm). Chromatographic conditions were 240 ℃ and 275 ℃ for the injector and detector ports, respectively. The oven temperature programme was initially set at 50 ℃ (5 min) and then increased to 170 ℃ at 5 ℃/min, where it remained for 31 min and then increased at a rate of 15 ℃/min to 230 ℃, where it remained for the last 20 min. The carrier gas was hydrogen.

Bioinformatic processing of 16S rRNA gene sequencing data

Demultiplexed paired-end reads from 16S rRNA gene sequencing were first checked for quality using FastQC [26] for an initial assessment. Forward and reverse paired-end reads were joined into single reads using the C++program SeqPrep [27]. After joining, reads were filtered for quality based on: (i) maximum three consecutive low-quality base calls (Phred < 19) allowed; (ii) fraction of consecutive high-quality base calls (Phred > 19) in a read over total read length ≥ 0.75; (iii) no "N" -labeled bases (missing/uncalled) allowed. Reads that did not

match all the above criteria were excluded. All remaining reads were combined in a single FASTA file for the identification and quantification of OTUs (operational taxonomic units). Reads were aligned against the Silva database v.132 for closed-reference identification, with 97% cluster identity [28], applying the CD-HIT clustering algorithm [29]. A predefined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, genus). By counting the abundance of each OTU, the OTU table was created and then grouped at each phylogenetic level. Records belonging to OTUs with total counts lower than 10 in fewer than 2 samples were filtered out. Prior to all subsequent analyses, the filtered OTU table was normalised for uneven amplification and sequencing depth by cumulative sum scaling (CSS) [30] All of the above steps, except the FastQC reads quality check, were performed with the QIIME 1.9 open-source bioinformatics pipeline for microbiome analysis [31].

Alpha- and beta-diversity indices

The microbial diversity of the different samples was assessed within- (alpha-diversity) and across- (beta-diversity) samples. The following alpha-diversity indices were estimated from the OTU table for the three dairy cow microbiomes: abundance-based coverage estimator (ACE), Chao1, Fisher's alpha, Shannon and Simpson, alongside the simple number of observed OTUs. Details on the calculation of these indices can be found in Biscarini et al. (S2 Appendix) [32]. The across-sample microbiota (beta) diversity was quantified by calculating Bray–Curtis dissimilarities [33].

Statistics and bioinformatics data analysis

All data related to microbiological analysis are presented as means±standard error (SE). Differences among the bacteriological counts were calculated by one-way ANOVA using Minitab ver. 14.13 (Minitab Inc.). For each of the three considered cow microbiomes, differences between experimental groups (10 cows for treatment and for each timepoint) in terms of alpha diversity indices, F:B ratios and OTU counts were evaluated within timepoint using a linear model of the following form:

$$y_{ij} = \mu + \text{treatment}_j + e_{ij} \qquad (A)$$

where y_{ij} are individual alpha-diversity indices, F:B ratios or normalised OTU counts for sample *i* belonging to treatment group *j*; treatment *j* is the effect of treatment as categorical effect (control, sealant, aloe); and e_{ij} are the model residuals.

As for beta-diversity, Bray–Curtis distances between groups along timepoints were evaluated non-parametrically using the permutational analysis of variance (PERMANOVA) approach with 999 permutations [34].

To understand the association between the volatile organic compounds produced by bacteria, all the variables need to be considered simultaneously, in order to highlight the relationships with the various metabolic processes. Unfortunately, many of the analyzed fatty acids have mutual correlations, which generate a redundant information and create difficulties in interpretation. We used principal component analysis (PCA) to achieve this goal. The PCA is a multivariate dimension reduction technique principally aimed at synthesizing information contained in a set of n observed variables (y_1, ..., y_n) by seeking a new set of p (p<n) variables (X_1, ..., X_p), named principal components (PCs). The first PC (PC1) explains most of the variability, whereas the remaining PCs (PC2, PC3, ..., PCn, n=number of variables) account for the remaining variability in the data. Each PC is independent and orthogonal to the others. Generally, the first few PCs are sufficient to describe most of the total data variations [35].

Reads from 16S rRNA gene sequencing were processed with the QIIME 1.9 pipeline [29], used also to estimate most diversity indices. The ACE index and sample-based rarefaction were estimated using own Python (https://github.com/filippob/Rare-OTUs_ACE.git) and R (https://github.com/filippob/sampleBasedRarefaction) scripts. PERMANOVA of Bray-Curtis distances was carried out with the R package Vegan [36]. The plots were generated using the ggplot2 R package [37]. Additional data handling and statistical analysis were performed with the R environment for statistical computing (R Core Team R) [38].

Results

Microbiological analysis

As reported in Supplementary File - Tables 1 and 2, *Aloe arborescens* administration did not affect the bacterial content of the raw milk and faecal samples of the three cow groups considered (Control, Sealant and Aloe). Regardless of the experimental group, an increase of about 1 log was observed in all bacterial groups (TMB, psychrotrophic bacteria, Enterobacteriaceae, *Escherichia coli*, LAB and enterococci) from the colostrum to the mature milk (T4).

Alpha-diversity

Figure 1 shows the distribution of the alpha-diversity indexes estimated for the rumen, rectum and milk microbiomes in the three experimental groups (Control, Sealant, Aloe) at different timepoints (more details on the alpha-diversity indices can be found in Supplementary File - Table 3). From equation [A], no

statistically significant differences (significance threshold: p-value<0.05) between the three experimental groups (Control, Sealant and Aloe) were found at any timepoint in the rumen microbiota. Statistically significant differences (p-value<0.05) in alpha-diversity among the three experimental groups were observed in the rectum (at T2 and T3) and milk (T1 and T4) microbiomes. Notably, the number of significant differences between treatments increased with time: from zero at T1 to six at both T2 and T3 in the rectum microbiome, and from two at T1 to five at T4 in the milk microbiome. This is consistent with the effect of the experimental treatment over time, after a lag necessary for the biological effect to be exerted. Specifically, an increased microbial diversity was observed with the *Aloe arborescens* treatment, especially in the milk microbiota (p-values for all comparisons are reported in Supplementary File - Table 5).

Beta-diversity

Figure 2 reports the first two dimensions from the non-metric multidimensional scaling of Bray-Curtis dissimilarities in the three dairy cow microbiomes at different timepoints. In the rumen microbiome (Fig. 2A) no clear clustering by treatment was observed (non-significant p-value from PERMANOVA). In the rectum microbiome (Fig. 2B), the distances between treatment groups were significantly different (p-value<0.01), especially at T3. In the milk microbiota (Fig. 2C), a statistically significant (p-value<0.01) difference between treatments was observed.

Firmicutes to Bacteroidetes ratio

Figure 3 reports the F: B (Firmicutes to Bacteroidetes) ratio over time for the three experimental groups and the different cow microbiomes. The F: B ratio showed a significant increase at T3 in the rectum microbiome for both the Sealant and Aloe (p-values 0.0402 and 0.0156)
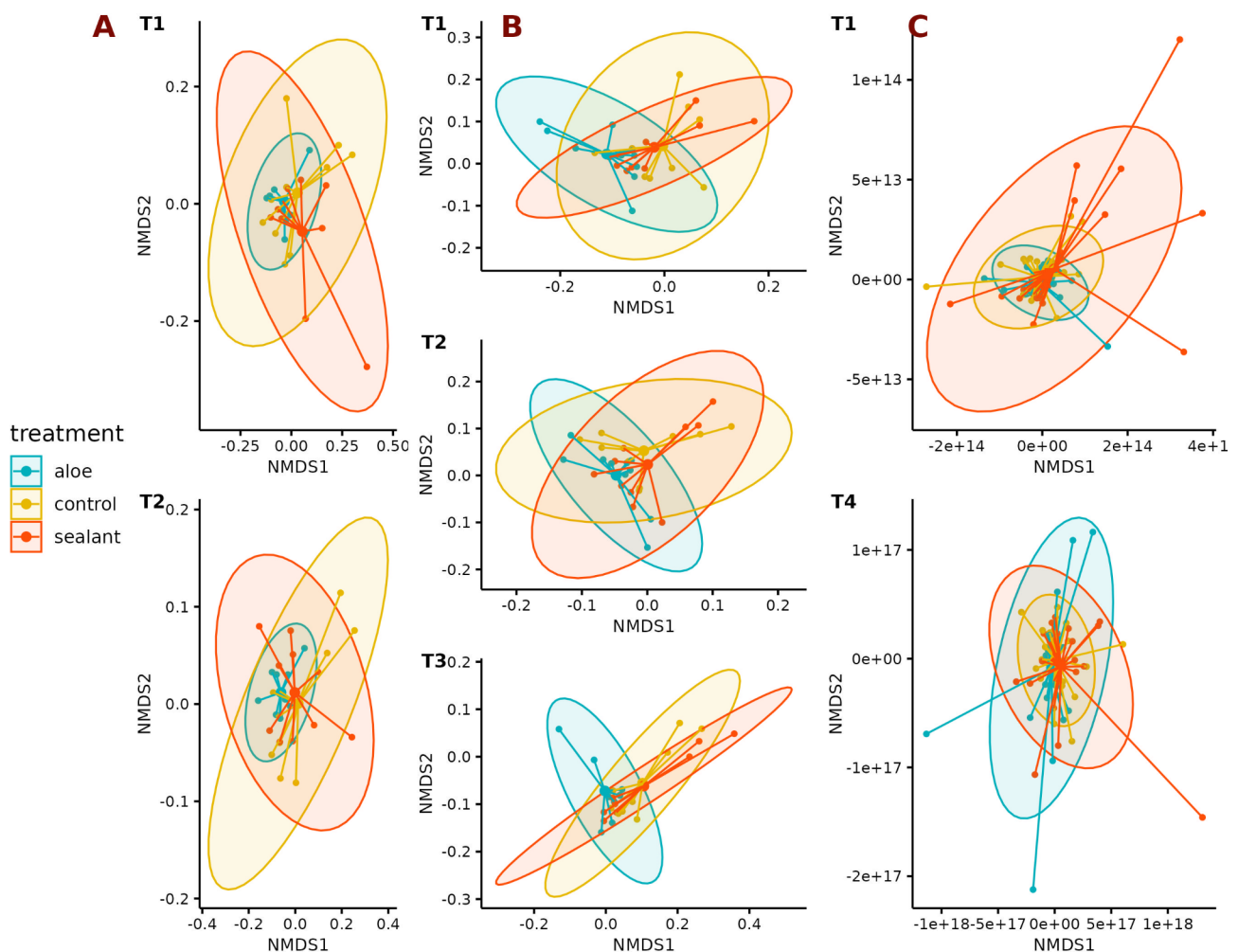

Fig. 2 Beta-diversity analysis: first two dimensions from the (non-metric) multi-dimensional scaling of the Bray-Curtis dissimilarity matrix. Results were grouped by experimental units: by microbiota (rumen (**A**), rectum (**B**), milk (**C**) samples), timepoints (T1, T2, T3, T4) and treatments (Aloe, Control, Sealant groups)
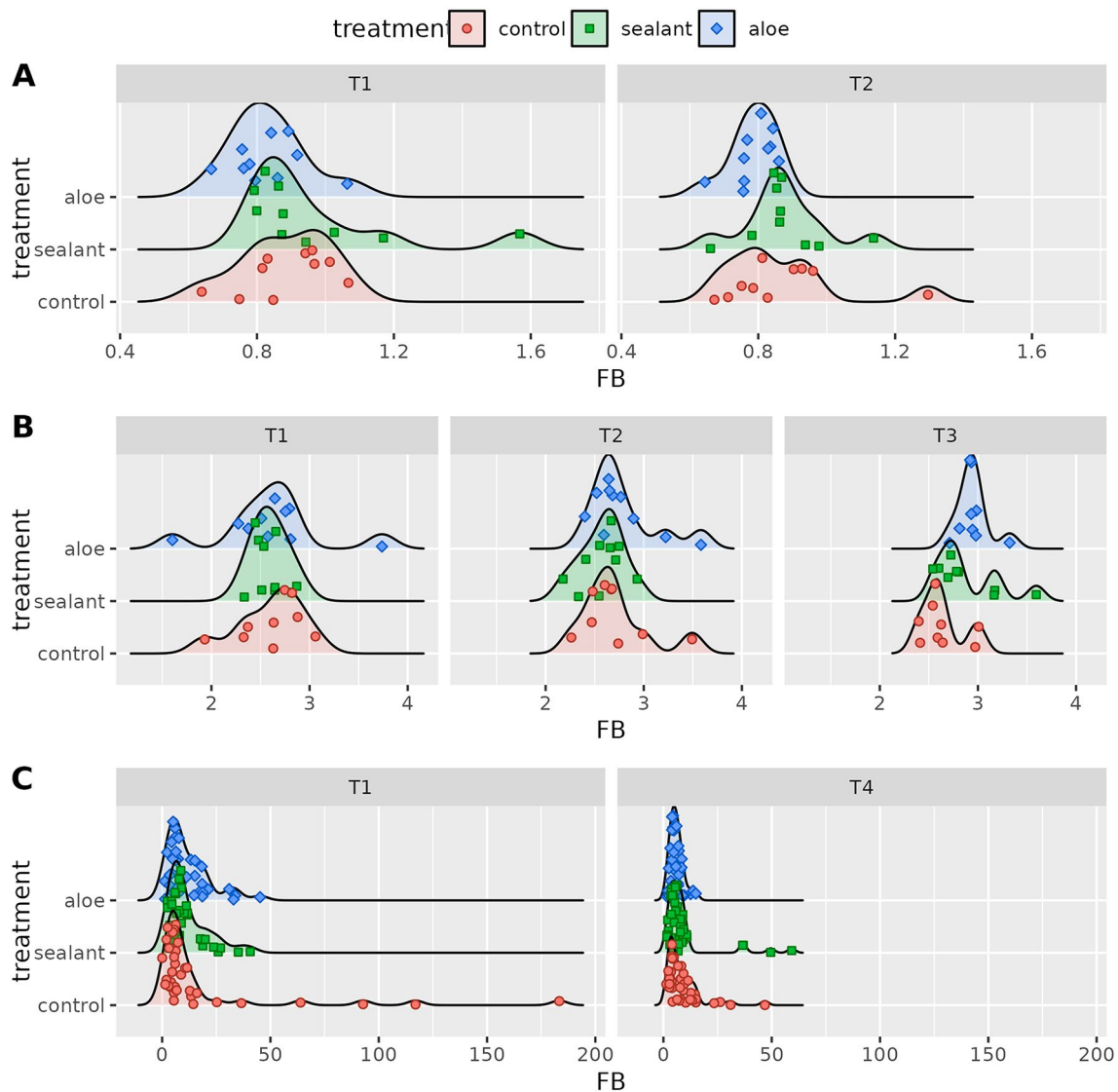
Fig. 3 Distribution of the F: B ratio in the rumen liquor (**A**), rectum (**B**) and milk (**C**) microbiomes between treatments at each timepoint

treatments with respect to the Control group. All other F: B differences (rumen and milk microbiomes, other timepoints) were not significantly larger than zero.

Taxonomic characterization of rumen, rectum and milk samples

The 16S rRNA gene sequencing data were further used to estimate the relative abundance of microbial taxonomic groups in the rumen liquor, rectum and milk microbiomes. A comprehensive list of the microbial phyla is provided in Supplementary File – Table 4.

From a descriptive standpoint, as illustrated in Fig. 4, more than 80% of the microbial composition in the rumen (Fig. 4A) consisted of Bacteroidetes (average relative abundance: 41%), Firmicutes (36%) and Proteobacteria (4%). Regardless of the treatments applied, Bacteroidetes increased in relative abundance (∼2.5%) from

T1 to T2, while the relative abundance of Firmicutes and Proteobacteria decreased (∼ 2%).

For the rectum microbiota, most of the reads belonged to Firmicutes (summing up to about ∼65% on average relative abundance) followed by Bacteroidetes (∼ 25%); the remaining 10% was composed by subdominant phyla as Proteobacteria, Verrucomicrobia, Tenericutes, Cyanobacteria (Fig. 4B). From T1 up to T3, respect to the Sealant group, the Aloe and Control groups revealed an increase of Firmicutes and a reduction of the relative abundances of Bacteroidetes.

A comparison between the Sealant and Control groups showed that 11 OTUs were significantly different (p-value<0.05) at T2, and 29 OTUs at T3. Out of the 29 OTUs, 19, including the genera Ruminococcaceae UCG-010 and Bacteroides, exhibited a slightly positive difference, while Alloprevotella and Prevotellaceae UCG-001
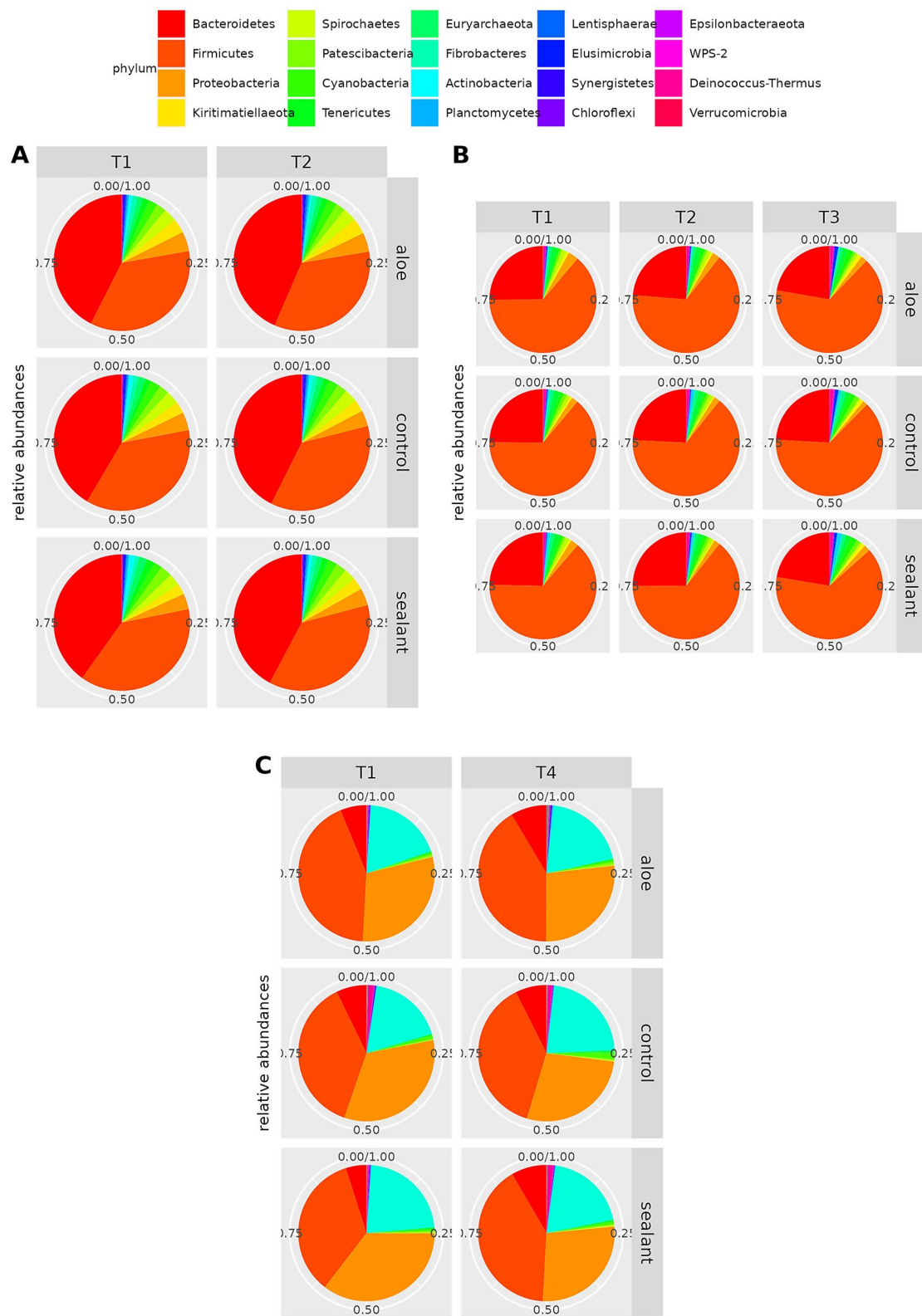
Fig. 4 Relative abundances of the taxa (≥ 1%) in the rumen liquor (**A**), rectum (**B**) and milk (**C**) microbiota (OTUs shared by at least 95% of the samples), grouped by taxonomic phylum level

showed a statistically significant negative difference compared to Control group. In the comparison between Aloe and the Control group, 11 OTUs were significantly different (p-value<0.05) at T2. Only Succinivibrio displayed a positive difference (Fig. 6, and Supplementary File Excel). At T3, 36 OTUs were found to be different, with only *Clostridium* in sensu stricto 1 showing a negative difference compared to the Control group (Fig. 6). The remaining 35 genera, including Alistipes, Ruminococcaceae UCG-010, Ruminococcaceae UCG-014, Ruminococcaceae UCG-013, Ruminococcaceae UCG-005, Alloprevotella, Lachnospiraceae NK4A136 group, Lachnospiraceae AC2044 group and Bacteroides, increased compared to Control group.

Before treatment (T1), Firmicutes (with an average relative abundance of 40%), Proteobacteria (∼30%), Actinobacteria (∼20%) and Bacteroidetes (∼6%) comprised the four predominant phyla collectively representing over 95% of the entire milk microbiota (Fig. 4C). This distribution aligns with the composition of the healthy milk microbiome as previously documented in literature [39], or mentioned as the main constituents of dairy cows' milk microbiota, regardless of the mammary gland status [40]. At T4, an increase in Bacteroidetes (∼8.5%) and a decrease in Proteobacteria (∼27%) were observed across all experimental groups, regardless of treatment. Additionally, there was an increase in the Actinobacteria phylum for the Aloe (+1.5%) and Control (+5%) groups and a reduction for the Sealant (-3%) group. Finally, Firmicutes exhibited a reduction in relative abundance for the Aloe (-1%) group, an increase (+6%) in the Sealant group, and remained stable for the Control group.

At the genus level, differences were identified when analysing the two treatments compared to the Control group. In the Sealant group, at T4, 93 OTUs showed statistically significant differences (p-value<0.05): 69, including *Corynebacterium* 1, *Brevibacterium*, *Escherichia-Shigella*, *Staphylococcus*, Ruminococcaceae UCG-005, *Psychrobacter*, and *Pelomonas*, displayed a positive difference respect to the Control group, while 24 exhibited a statistically significant negative difference, including *Akkermansia* and Rikenellaceae RC9 gut group, with higher diversity (Fig. 5, Supplementary File Excel).

In the Aloe group, 96 OTUs resulted significantly different: among the 58 with a positive difference compared to the Control group, *Staphylococcus* spp, *Brevibacterium*, *Corynebacterium* 1, *Atopostipes* and Bacteroides had the highest values. Conversely, among the 38 OTUs with a negative difference, *Escherichia-Shigella*, Ruminococcaceae NK4A214 group, *Akkermansia*, Rikenellaceae RC9 gut group, *Pedomonas*, *Pelomonas* and *Pransereulla* were the most prevalent (Fig. 5, Supplementary File Excel).

Volatilome results

From the analysis of the rumen volatilome, 40 compounds from the classes of acids, alcohols, sulfur compounds, aromatic compounds and terpenes were identified. Among these, the principal component analysis (PCA) focused on the 16 most significant compounds (Fig. 6), revealing two distinct groups. One group comprised volatile fatty acids (VFA), while the other included compounds from the feed. The remaining compounds did not exhibit apparent relationships with each other.

Furthermore, the distribution of the samples did not display groupings associated with the treatments; in some instances, variability attributed to the individual animals was evident. Specifically, there was no observable effect of Aloe treatment on the volatilome.

Milk fatty acids

The analysis of milk fatty acids aimed to investigate the impact of administering *Aloe arborescens* at the doses specified in this study on the acidic composition of milk fat. The results of the analysis do not show significant differences between timing T1 and T4 for all fatty acids except for the C18:2c9t11 (CLA) (p-value≤0.01) and the C10:1 (p-value≤0.05) that are present in slightly lower amounts following Aloe administration as reported in Table 1.

Discussion

The dry-off period is a physiological phase marked by significant changes in the metabolism of dairy cows, leading to alterations in metabolism and liver function. The supplementation of lyophilized *Aloe arborescens* appears to enhance adaptation to the dry period on two fronts: at the rumen level and by modulating specific metabolic pathways. The RABOLA project was the first scientific effort to study rumen fermentation, milk production and blood biomarkers upon in vivo supplementation with *Aloe* spp. during dry-off in dairy cows that did not receive antibiotic therapy but were administered only an internal teat sealant instead [15, 41]. The project results revealed altered proportions of volatile fatty acids (VFA) and a reduction in dry fecal content. Interestingly, the subsequent lactation showed an ameliorated inflammatory status and increased milk yield, while milk composition, SCC, and mastitis incidence remained unaffected by *Aloe* treatment. In the context of the same project, in this work we examined the effects of lyophilized *Aloe arborescens* dietary supplementation on the bacterial components by analyzing the microbiomes of the rumen, the rectum and the milk in thirty cows partly included in the previous reports [15]. Our aim was to obtain a deeper understanding of whether *Aloe* supplementation influences the dynamics of relevant microbial populations of dairy cows.
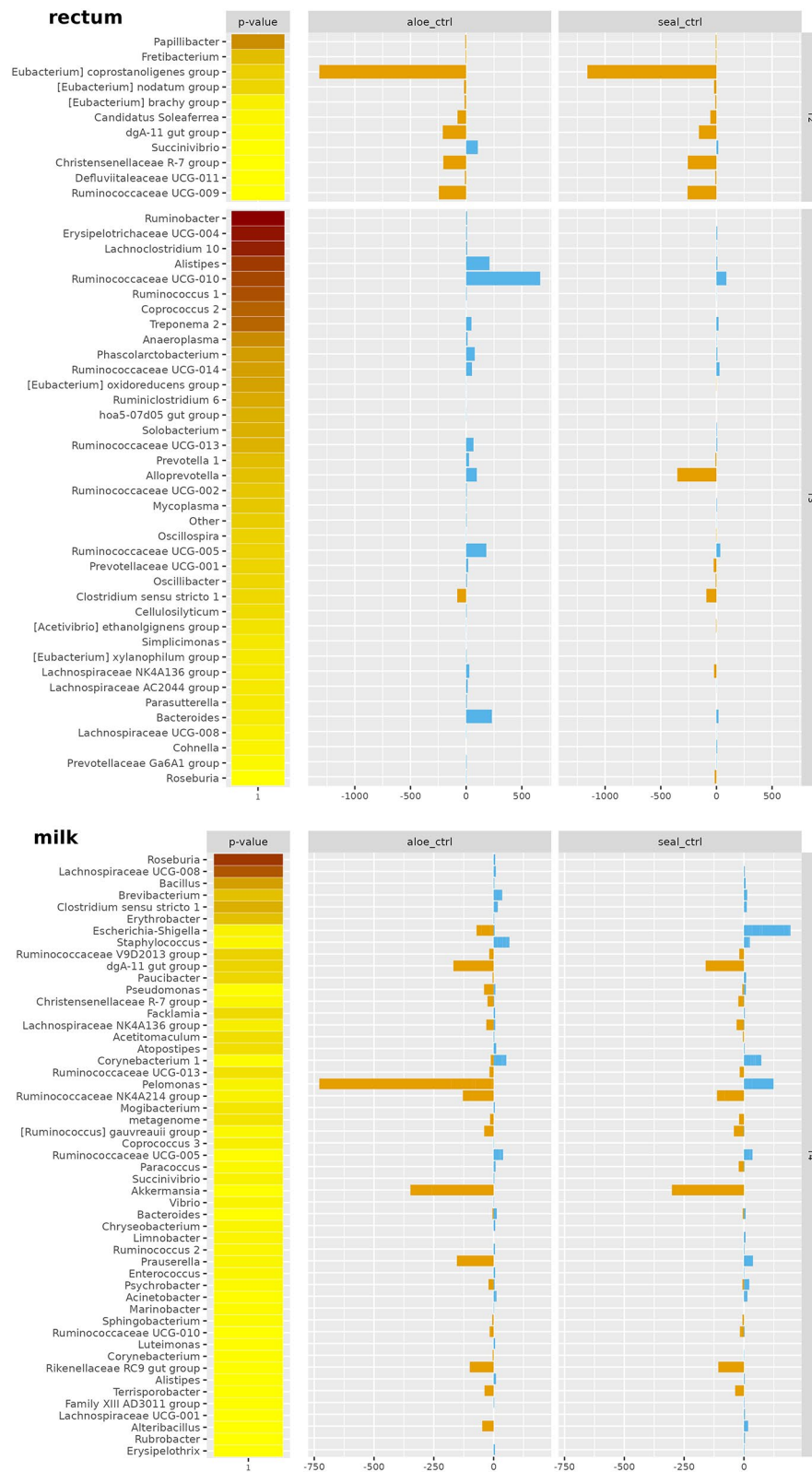
Fig. 5 Significantly different OTUs. OTU significantly different between groups (Aloe *versus* Control, Sealant *versus* Control), for rectum and milk microbiomes, from analysis of variance based on normalised counts. Only OTUs with per-group normalised counts > 2 and with annotated taxonomy (not 'uncultured') are reported
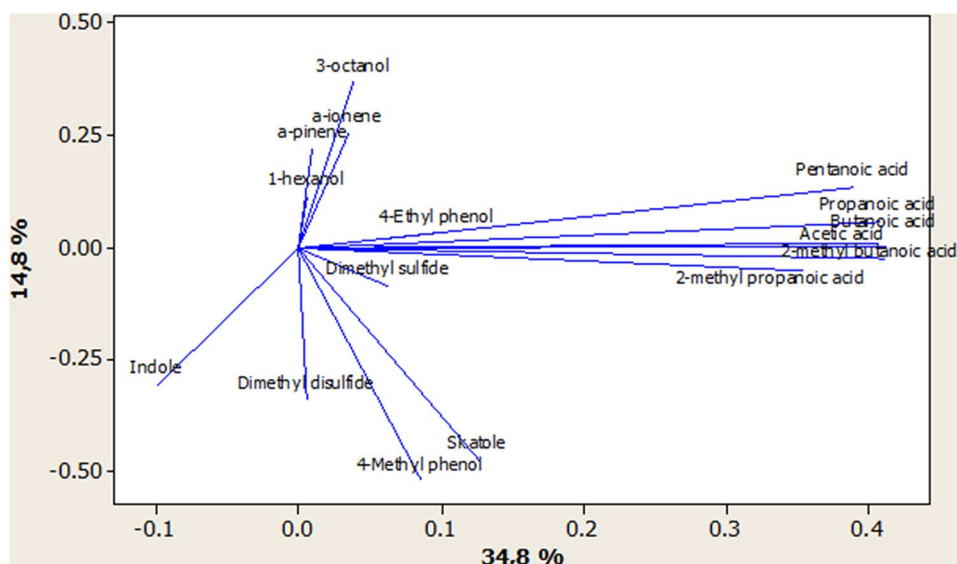
Fig. 6 Loading plot of the main organic compounds found in the rumen liquor in cows

Bacteriology of milk and faecal samples

Aloe arborescens administration did not affect the bacterial content of the raw milk and faecal samples of the three cow groups considered (Control, Sealant and Aloe). On the other hand, our findings highlighted that the lactation stage may influence the abundance of some bacterial groups in raw milk. In fact, we observed a one-log increase of the level of total bacterial count, psychrotrophic bacteria, LAB and enterococci from colostrum to mature milk. The lowest loads of microorganisms in milk samples at calving could be related to the strong antimicrobial activity of bovine colostrum, which may hinder the growth of Gram-positive and Gram-negative bacteria [42]. To our knowledge, no data are available on the milk microorganism content in relation to the cow lactation stage, but similar results were obtained by Khodayar-Pardo et al. [43] in human breast milk.

Effect of Aloe on the rumen microbiome

The rumen microbiota after a week of *Aloe* supplementation was not found to vary among treatments (after adjusting for the timepoint) revealing the presence of Bacteroidetes and Firmicutes as the prevailing phyla, a common finding from cattle rumen microbiota [12, 44, 45]. No variations in composition and abundance of OTUs were detected among the rumen microorganisms, confirming the absence of effects of the *Aloe* treatment on rumen fermentation and feed digestibility, as previously observed by adding *Aloe arborescens* homogenate to rumen liquor during in vitro experiments [12]. Our results were further supported by the volatilome analysis, which showed no observable major effect on rumen liquor composition after this diet supplementation. This lack of impact can be attributed to the fact that the

samples were analyzed only seven days post treatment. In fact, the plasma metabolome [41] reveals that *Aloe* has positive effects when supplemented 7 days before and after dry-off on liver function and have modulatory effect on rumen fermentation.

It is also worth considering that the rumen and lower gut microbiomes are different both in composition and function due to their respective environments. The rumen microbiota is rich in bacteria that degrade fibrous feed through fermentation, producing volatile fatty acids (VFAs), essential amino acids, and vitamins. In contrast, the lower gut microbiome contains bacteria with a greater ability to absorb nutrients. Due to the wider variety of substrates available for fermentation, the microbiome of ruminal fluid exhibits greater diversity compared to that of feces. Consequently, the different compositions of the bacterial populations may explain the varying responses to dietary supplementation with *Aloe arborescens* [46, 47].

Effect of Aloe on the rectum microbiome

As expected, the composition of the rectum microbiota is influenced by diet and feed supplementation. Firmicutes typically enhance the availability of nutrients, whereas Bacteroidetes are less energetically favorable to the host [48]. Alterations in diet have a notable impact on the Firmicutes: Bacteroidetes ratio [49]; our findings indicate an elevated presence of Firmicutes and a reduction in Bacteroidetes in rectum samples from cows that received *Aloe arborescens* supplementation. Following Aloe supplementation, there was an increase in the abundance of bacteria from the *Alistipes* spp., Ruminococcaceae UCG-014, UCG-010, UCG-013 and UCG 005 groups, Prevotellaceae and Bacteroides genera. Ruminococcaceae spp.,

Table 1 Fatty acid (FA) profile (g/100 g of fatty acid) in milk of cows before (T1) and after (T4) administration of *Aloe arborescens*

Fatty Acid	T1	T4	SE	*p*-value
C4:0	3.47	3.57	0.40	ns
C6:0	2.27	2.13	0.20	ns
C8:0	1.31	1.22	0.11	ns
C9:0	0.05	0.03	0.02	ns
C10:0	2.98	2.83	0.34	ns
C10:1	0.37	0.29	0.06	*
C11:0	0.08	0.07	0.04	ns
C12:0	3.55	3.47	0.50	ns
C13:0 anteiso	0.11	0.10	0.06	ns
C12:1	0.12	0.10	0.03	ns
C13:0	0.13	0.11	0.04	ns
C14:0 iso	0.14	0.14	0.03	ns
C14:0	10.83	10.66	0.90	ns
C15:0 iso	0.27	0.26	0.03	ns
C15:0 anteiso	0.51	0.50	0.10	ns
C14:1	1.51	1.30	0.26	ns
C15:0	1.25	1.30	0.24	ns
C16:0 iso	0.32	0.33	0.06	ns
C15:1	0.03	0.02	0.01	ns
C16:0	35.71	35.00	1.86	ns
C17:0 iso	0.27	0.25	0.07	ns
C17:0 anteiso	0.20	0.25	0.09	ns
C16:1	2.60	2.51	0.26	ns
C17:0	0.49	0.55	0.07	ns
C17:1	0.22	0.24	0.05	ns
C18:0	8.09	8.75	1.36	ns
C18:1t9	0.33	0.37	0.10	ns
C18:1t11	0.71	0.91	0.19	ns
C18:1c9	19.02	20.09	2.00	ns
C18:2tt	0.26	0.25	0.07	ns
C18:2n6	1.96	1.78	0.28	ns
C18:3n3 ALA	0.54	0.41	0.13	ns
C18:2c9t11 CLA	0.31	0.21	0.06	**
TOT FA%	100	100		

ns = not significative; * = p-value < 0.05; ** = p-value < 0.01; ALA = a-linolenic acid; CLA = Conjugated Linoleic Acid

together with the Lachnospiraceae spp., which showed a slight increase in our study for NK4A136 and AC2044 groups, belong to the order Clostridiales and are bacteria that degrade cellulose and hemicellulose. These bacteria contribute to the enhancement of animal energy metabolism by producing β-hydroxybutyrate and acetoacetate, which serve as energy substrates for epithelial cells [50]. This process helps to improve the overall energy metabolism of dairy cows, maintain a healthy state of the gut and might also improve the feed efficiency [15, 41, 51, 52].

Aloe supplementation also increased the abundance of *Prevotella* spp. and *Alistipes* spp. Previous researches revealed that various *Prevotella* spp. can selectively utilize carbohydrates and proteins from the diet, leading to the production of succinate and acetate [53].

Additionally, one of their significant roles is in the protein and peptide breakdown [54]. This genus also plays a crucial role in normal intestinal metabolism and contributes to maintaining intestinal health [55].

Alistipes spp. are classified as a member of the phylum Bacteroidetes, belonging to the Rikenellaceae family, primarily found in the healthy human gastrointestinal tract microbiota [56]. Regarding human pathogenicity, there is conflicting evidence suggesting that *Alistipes* spp. may have protective effects against specific dysbiosis and diseases, such as liver fibrosis, colitis, cancer immunotherapy, and cardiovascular disease. On the contrary, other studies indicate that *Alistipes* is pathogenic in colorectal cancer and is associated with mental signs of depression [56]. Meanwhile, changes in diet have been shown to affect the abundance of *Alistipes*, which is a bile-tolerant bacterium [57]. In cow, *Alistipes* spp. constitute a group strongly linked to the rectum microbiota [58], representing 8.7% of the large intestine microbiota in dairy cows. Their bile tolerance seems to play an important role in the rectum niche [59], promoting health-related phenotypes [60].

Conversely, the Sealant group, under identical experimental conditions except for Aloe supplementation, exhibited a rectum microbiome with a relatively low increase in Ruminococcaceae UCG-010 group and a significant reduction in Alloprevotella and Prevotellaceae UCG-001 group abundance compared to Control group. These genera are commonly reported to be among the most abundant in the rectum microbiota of Holstein cows [61, 62].

Effect of Aloe on the milk microbiome

Aloe spp. is well-known for its antimicrobial properties [63, 64]. It has been previously employed as an intra-mammary remedy for treating mastitis in organic systems [65]. Additionally, it has been utilized in combination with *Weissella cibaria*, a probiotic bacterium, to develop a teat bio-sealant as a novel approach to prevent infections [66]. In our study, *Aloe arborescens* was used as dietary supplement for dairy cows at dry-off: in previous studies, it had been already used in the transition period where positive effects on inflammometabolic response, milk composition and mammary health were observed [67, 68]. Aloe feed supplementation prior to dry-off appeared to influence the milk microbial composition at the onset of the following lactation in the Aloe group compared to the Control and Sealant groups. Specifically, there was a decrease in *Escherichia-Shigella*, *Akkermansia* and *Pelomonas* genera, while an increase was observed in *Staphylococcus* spp., *Atopostipes* and Bacteroides genera. As reported by Zhu et al. [69], *Staphylococcus*, especially *S. aureus* and coagulase-negative *Staphylococcus*, was among the most prevalent genera in

studies on both human and cow milk microbiota [70–72]. Some non-aureus species, such as *S. chromogens*, *S. simulans*, *S. xylosus*, are often detected in bovine milk and are associated with preventing mastitis pathogen infections by producing bacteriocin [73]. Furthermore, as facultative anaerobes, *Staphylococcus* can participate in lactate metabolism, colonize the gastrointestinal tract, and contribute to the colonization of strict anaerobes by consuming O_2 [73, 74]. Additionally, the high abundance of Bacteroides in milk samples from lactating cows suggests its role for the development of calves' immunity [75].

Differing from previous studies [76, 77], the Sealant group revealed higher diversity compared to the Control group across all alpha-diversity indices. However, it is worth noting that the main families within the Sealant group that experienced an increase were *Escherichia-Shigella*, *Pelomonas* and *Corynebacterium* 1 genera, which are commonly reported as microorganisms associated with mastitis. As expected, in the Control group, where a combination of antibiotics and teat sealant was applied, there was a decrease in the diversity of the milk microbiota.

The data of the present work have also demonstrated that the fatty acid profile of milk is not influenced by the *Aloe arborescens* supplementation in the diet. To the best of our knowledge, this is the first time this assessment has been made in bovine milk.

As can be seen from Table 1, the presence of *Aloe arborescens* in the diet did not have any effect on rumen lipid metabolism, in fact all branched fatty acids (BCFA) did not show significant differences. These fatty acids are synthesized by ruminal bacteria, and it has been demonstrated in several works that their profile in milk represents a valid indicator of ruminal metabolic activity [78–80]. The lack of significant differences in BCFA is an indication that *Aloe arborescens* does not modify the lipid metabolism of ruminal bacteria.

The same consideration must be made for the fatty acids involved in the ruminal biohydrogenation process. Table 1 shows that C18:1t9, C18:1t11, C18:2t showed no significant differences after supplementation with *Aloe arborescens*. These fatty acids are the intermediate products of the biohydrogenation of linoleic and α-linolenic acids by ruminal bacteria [80]. Vaccenic acid (C18:1t11), in particular, is the precursor of the mammary synthesis of rumenic acid (C18:2c9t11), the main conjugated isomer of Linoleic Acid (CLA) [81]. Although C18:1t11 showed no significant differences, we observed a reduction in C18:2c9t11 content. The reason may be due to a reduction in the activity of Stearoyl CoA Desaturase (SCD), the enzyme responsible for the insertion of a double bond in position Δ9 [82]. This hypothesis is confirmed by the significant reduction of C10:1 and of C14:1c9 which however did not show significant

difference. These fatty acids are also the products of SCD activity [81] and this indicates an effect of Aloe treatment on breast metabolism.

Conclusions

This study provides insights into the bacterial community composition of rumen, rectum and milk of dairy cows in relation to SDCT when replacing the prophylactic use of antibiotics with internal teat sealants and oral administration with lyophilized *Aloe arborescens*. The potential biological benefits of oral administration with Aloe have been presented and discussed. In general, we observed that both treatments do not significantly alter the rumen, rectum and milk microbiomes of healthy cows, making them good candidates for SDCT. As expected, the few effects observed were mainly positive, in the rectum -as well as in the milk- microbiome, involving microorganisms that usually contribute to the enhancement of animal energy metabolism or have a protective effect against enteric dysbiosis and diseases. Since samples were analyzed only seven days' post-treatment, further studies will be needed to better understand if *Aloe arborescens* supplementation has effect on rumen fermentation and feed digestibility over a longer time period.

Declarations

Ethics approval and consent to participate
The trial was carried out at Università Cattolica del Sacro Cuore research dairy barn (Cerzoo, San Bonico, Piacenza, Italy) in accordance with Italian laws on animal experimentation and ethics (Italian Health Ministry Authorisation No. 464/2019-PR in agreement with D. Lgs. no. 26, 04/03/2014), as described in Cattaneo et al. (2022).

Consent for publication
N/A.

Competing interests
The authors declare no competing interests.

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References
1. Scherpenzeel CGM, Tijs SHW, den Uijl IEM, Santman-Berends IMGA, Velthuis AGJ, Lam TJGM. Farmers' attitude toward the introduction of selective dry cow therapy. J Dairy Sci. 2016;99(10):8259–66. https://doi.org/10.3168/jds.2016-11349.
2. Niemi RE, Hovinen M, Vilar MJ, Simojoki H, Rajala-Schultz PJ. Dry cow therapy and early lactation udder health problems-associations and risk factors. Prev Vet Med. 2021;188:105268. https://doi.org/10.1016/j.prevetmed.2021.105268.
3. van Hoeij RJ, Lam TJGM, Bruckmaier RM, Dijkstra J, Remmelink GJ, Kemp B, van Knegsel ATM. Udder health of dairy cows fed different dietary energy levels after a short or no dry period without use of dry cow antibiotics. J Dairy Sci. 2018;101(5):4570–85. https://doi.org/10.3168/jds.2017-13448.
4. Pruden A, Larsson DG, Amézquita A, Collignon P, Brandt KK, Graham DW, Lazorchak JM, Suzuki S, Silley P, Snape JR, Topp E, Zhang T, Zhu YG. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Envir Health Perspect. 2013;121(8):878–85. https://doi.org/10.1289/ehp.1206446.
5. Oliver JP, Gooch CA, Lansing S, Schueler J, Hurst JJ, Sassoubre L, Crossette EM, Aga DS. Invited review: fate of antibiotic residues, antibiotic-resistant bacteria, and antibiotic resistance genes in US dairy manure management systems. J Dairy Sci. 2020;103(2):1051–71. https://doi.org/10.3168/jds.2019-16778.
6. Lopreiato V, Mezzetti M, Cattaneo L, Ferronato G, Minuti A, Trevisi E. Role of nutraceuticals during the transition period of dairy cows: a review. J Anim Sci Biotechnol. 2020;11:96. https://doi.org/10.1186/s40104-020-00501-x.
7. Ingvartsen KL, Moyes K. Nutrition, immune function and health of dairy cattle. Animal. 2013; 7 Suppl 1:112–22. https://doi.org/10.1017/S175173111200170X
8. Trevisi E, Minuti A. Assessment of the innate immune response in the periparturient cow. Res Vet Sci. 2018;116:47–54. https://doi.org/10.1016/j.rvsc.2017.12.001.
9. Minuti A, Bionaz M, Lopreiato V, Janovick NA, Rodriguez-Zas SL, Drackley JK, Loor JJ. Prepartum dietary energy intake alters adipose tissue transcriptome profiles during the periparturient period in Holstein dairy cows. J Anim Sci Biotechnol. 2020;11:1. https://doi.org/10.1186/s40104-019-0409-7.
10. Akaberi M, Sobhani Z, Javadi B, Sahebkar A, Emami SA. Therapeutic effects of Aloe spp. in traditional and modern medicine: a review. Biomed Pharmacother. 2016;84:759–72. https://doi.org/10.1016/j.biopha.2016.09.096.
11. Radha MH, Laxmipriya NP. Evaluation of biological properties and clinical effectiveness of Aloe vera: a systematic review. J Tradit Complement Med. 2014;5(1):21–6. https://doi.org/10.1016/j.jtcme.2014.10.006.
12. Bani P, Grossi P, Lucini L, Pellizzoni M, Minuti A, Trevisi E. Administration of *Aloe arborescens* homogenate to cattle: interaction with rumen fermentation and gut absorption of aloin. Italian J Anim Sci. 2016;15:2233–240. https://doi.org/10.1080/1828051X.2016.1157007.
13. Martin CR, Osadchiy V, Kalani A, Mayer EA. The brain-gut-Microbiome Axis. Cell Mol Gastroenterol Hepatol. 2018;6(2):133–48. https://doi.org/10.1016/j.jcmgh.2018.04.003.
14. De Filippo C, Di Paola M, Ramazzotti M, Albanese D, Pieraccini G, Banci E, Miglietta F, Cavalieri D, Lionetti P. Diet, Environments, and Gut Microbiota. A Preliminary Investigation in Children Living in Rural and Urban Burkina Faso and Italy. Front Microbiol. 2017;8:1979. https://doi.org/10.3389/fmicb.2017.01979
15. Cattaneo L, Piccioli-Cappelli F, Minuti A, Trevisi E. Drying-off dairy cows without antibiotic therapy and orally supplemented with lyophilized Aloe arborescens: effects on rumen activity, immunometabolic profile, and milk yield. J Anim Physiol Anim Nutr (Berl). 2022. https://doi.org/10.1111/jpn.13777.
16. National Research Council. Nutrient requirements of dairy cattle. 7th ed. National Academies; 2001.
17. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 2004;36:808–12.
18. Ma ZY, Zhang XM, Wang R, Wang M, Liu T, Tan ZL. Effects of Chemical and mechanical lysis on Microbial DNA Yield, Integrity, and Downstream Amplicon Sequencing of Rumen Bacteria and Protozoa. Front Microbiol. 2020;11:581227. https://doi.org/10.3389/fmicb.2020.581227.
19. Cremonesi P, Castiglioni B, Malferrari G, Biunno I, Vimercati C, Moroni P, Morandi S, Luzzana M. Improved method for rapid DNA extraction of mastitis pathogens directly from milk. J Dairy Sci. 2006;89:163–9. https://doi.org/10.3168/jds.S0022-0302(06)72080-X.
20. Cremonesi P, Ceccarani C, Curone G, Severgnini M, Pollera C, Bronzo V, Riva F, Addis MF, Filipe J, Amadori M, Trevisi E, Vigo D, Moroni P, Castiglioni B. Milk microbiome diversity and bacterial group prevalence in a comparison between healthy Holstein Friesian and Rendena cows. PLoS ONE. 2018;13:e205054. https://doi.org/10.1371/journal.pone.0205054.
21. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci USA. 2011;108(Suppl1):4516–22. https://doi.org/10.1073/pnas.1000080107.
22. Battelli G, Scano P, Albano C, Cagliani LR, Brasca M, Consonni R. Modifications of the volatile and nonvolatile metabolome of goat cheese due to adjunct of non-starter lactic acid bacteria. LWT- Food Sci Technol. 2019;116:108576. https://doi.org/10.1016/j.lwt.2019.108576.
23. Cai L, Koziel JA, Davis J, Lo YC, Xin H. Characterization of volatile organic compounds and odors by in-vivo sampling of beef cattle rumen gas, by solid-phase microextraction, and gas chromatography–mass spectrometry–olfactometry. Anal Bioanal Chem. 2006;386(6):1791–802. https://doi.org/10.1007/s00216-006-0799-1.
24. Revello Chion A, Tabacco E, Giaccone D, Peiretti PG, Battelli G, Borreani G. Variation of fatty acid and terpene profiles in mountain milk and Toma piemontese cheese as affected by diet composition in different seasons. Food Chem 20101; 21(2):393–9. https://doi.org/10.1016/j.foodchem.2009.12.048
25. Christie WW. Preparation of Ester derivatives of fatty acids for chromatographic analysis. Adv Lipid Methodol. 1993: e111.
26. Andrews S, FastQC:. A Quality Control Tool for High Throughput Sequence Data. Babraham Bioinformatics. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc, 2010.
27. John JA. SeqPrep v1.1 - Tool for Stripping Adaptors and/or Merging Paired Reads With Overlap Into Single Reads. 2011. Available online at: https://github.com/jstjohn/SeqPrep
28. Glöckner FO, Yilmaz P, Quast C, Gerken J, Beccati A, Ciuprina A, Bruns G, Yarza P, Peplies J, Westram R, Ludwig W. 25 years of serving the community with ribosomal RNA gene reference databases and tools. J Biotechnol. 2017;261:169–76. https://doi.org/10.1016/j.jbiotec.2017.06.1198.
29. Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22:1658–9. https://doi.org/10.1093/bioinformatics/btl158.
30. Paulson JN, Stine OC, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. Nat Methods. 2013;10(12):1200–2. https://doi.org/10.1038/nmeth.2658.

31. Caporaso JG, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010. https://doi.org/10.1038/nmeth.f.303.

32. Biscarini F, Palazzo F, Castellani F, Masetti G, Grotta L, Cichelli A, Martino G. Rumen microbiome in dairy calves fed copper and grapepomace dietary supplementations: composition and predicted functional profile. PLoS ONE. 2018;13:e205670. https://doi.org/10.1371/journal.pone.0205670.

33. Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. Ecol Monogr. 1957;27(4):326–49.

34. Anderson MJ. Permutation tests for Univariate or Multivariate Analysis of Variance and Regression. Can J Fish Aquat Sci. 2001;58:626–39. https://doi.org/10.1139/f01-004.

35. Jolliffe I. Principal Component Analysis. 2002; 2 edition, Springer.

36. Oksanen J et al. 2022. _vegan: Community Ecology Package_. R package version 2.6-4, https://CRAN.R-project.org/package=vegan

37. Wickham H. 2016. Ggplot2: Elegant Graphics for Data Analysis; Springer: Berlin/Heidelberg, Germany, ISBN 3319242776.

38. R Core Team. 2023. R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

39. Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, Plaizier JC, Khafipour E. Invited review: Microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci. 2018;101(12):10605–25. https://doi.org/10.3168/jds.2018-14860.

40. Maity S, Ambatipudi K. Mammary microbial dysbiosis leads to the zoonosis of bovine mastitis: a one-health perspective. FEMS Microbiol Ecol. 2020;97(1):fiaa241. https://doi.org/10.1093/femsec/fiaa241.

41. Cattaneo L, Rocchetti G, Piccioli-Cappelli F, Zini S, Trevisi E, Minuti A. Impact of dry-off and lyophilized Aloe arborescens supplementation on plasma metabolome of dairy cows. Sci Rep. 2023;13:5256. https://doi.org/10.1038/s41598-023-31922-z.

42. Costa A, Sneddon NW, Goi A, Visentin G, Mammi LME, Savarino EV, Zingone F, Formigoni A, Penasa M, De Marchi M. Invited review: bovine colostrum, a promising ingredient for humans and animals—Properties, processing technologies, and uses. J Dairy Sci. 2023. https://doi.org/10.3168/jds.2022-23013.

43. Khodayar-Pardo P, Mira-Pascual L, Collado MC, Martínez-Costa C. Impact of lactation stage, gestational age and mode of delivery on breast milk microbiota. J Perinatol. 2014;34(8):599–605. https://doi.org/10.1038/jp.2014.47.

44. Calabro S, Musco N, Tudisco R, Grossi M, Calabrò V, Ferrara M, Cutrignelli MI, Infascelli F. Effect of *Aloe arborescens* on in vitro rumen fermentations. J Nutr Ecol Food Res. 2013.;1(2)2013:117–123. https://doi.org/10.1166/jnef.2013.1014

45. Morgavi DP, Kelly WJ, Janssen PH, Attwood GT. Rumen microbial (meta) genomics and its application to ruminant production. Animal. 2013; 7(s1):184–201. https://doi.org/10.1017/S1751731112000419 PMID: 23031271.

46. Monteiro HF, Zhou Z, Gomes MS, Peixoto PM, Bonsaglia EC, Canisso IF, Weimer BC, Lima FS. Rumen and lower gut microbiomes relationship with feed efficiency and production traits throughout the lactation of Holstein dairy cows. Sci Rep. 2022;12(1):4904. https://doi.org/10.1038/s41598-022-08761-5.

47. Liu JH, Zhang ML, Zhang RY, Zhu WY, Mao SY. Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows. Microb Biotechnol. 2016;9(2):257–68. https://doi.org/10.1111/1751-7915.12345.

48. Xu Q, Qiao Q, Gao Y, Hou J, Hu M, Du Y, Zhao K, Li X. Gut microbiota and their role in Health and metabolic disease of dairy cow. Front Nutr. 2021;8:701511. https://doi.org/10.3389/fnut.2021.701511.

49. Plaizier JC, Danesh Mesgaran M, Golder H, Khafipour E, Kleen JL, Lean I, Loor J, Penner G, Zebeli Q. Review: enhancing gastrointestinal health in dairy cows. Animal. 2018;12(s2):s399–418. https://doi.org/10.1017/S1751731118001921.

50. Wang Y, Zhao Y, Nan X, Wang Y, Cai M, Jiang L, Luo Q, Xiong B. Rumen-protected glucose supplementation alters fecal microbiota and its metabolic profiles in early lactation dairy cows. Front Microbiol. 2022;13:1034675. https://doi.org/10.3389/fmicb.2022.

51. Biddle A, Stewart L, Blanchard J, Leschine S. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity. 2013;5:627–40. https://doi.org/10.3390/d5030627.

52. Mancabelli L, Milani C, Lugli GA, Turroni F, Cocconi D, van Sinderen D, Ventura M. Identification of universal gut microbial biomarkers of common human intestinal diseases by meta-analysis. FEMS Microbiol Ecol. 2017;93(12). https://doi.org/10.1093/femsec/fix153.

53. Stevenson DM, Weimer PJ. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl Microbiol Biotechnol. 2007;75(1):165–74. https://doi.org/10.1007/s00253-006-0802-y.

54. Lourenco JM, Kieran TJ, Seidel DS, Glenn TC, Silveira MFD, Callaway TR, Stewart RL Jr. Comparison of the ruminal and fecal microbiotas in beef calves supplemented or not with concentrate. PLoS ONE. 2020;15(4):e0231533. https://doi.org/10.1371/journal.pone.0231533.

55. Xue MY, Sun HZ, Wu XH, Liu JX, Guan LL. Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. Microbiome. 2020;8(1):64. https://doi.org/10.1186/s40168-020-00819-8.

56. Parker BJ, Wearsch PA, Veloo ACM, Rodriguez-Palacios A. The Genus Alistipes: gut Bacteria with emerging implications to inflammation, Cancer, and Mental Health. Front Immunol. 2020;11:906. https://doi.org/10.3389/fimmu.2020.00906.

57. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 2014;505(7484):559–63. https://doi.org/10.1038/nature12820.

58. Holman DB, Gzyl KE. A meta-analysis of the bovine gastrointestinal tract microbiota. FEMS Microbiol Ecol. 2019;95(6):fiz072. https://doi.org/10.1093/femsec/fiz072.

59. Lin L, Lai Z, Yang H, Zhang J, Qi W, Xie F, Mao S. Genome-centric investigation of bile acid metabolizing microbiota of dairy cows and associated diet-induced functional implications. ISME J. 2023;17(1):172–84. https://doi.org/10.1038/s41396-022-01333-5.

60. Zhang F, Zhao Y, Wang Y, Wang H, Nan X, Guo Y, Xiong B. Dietary supplementation with calcium propionate could beneficially alter rectal microbial composition of early lactation dairy cows. Front Vet Sci. 2022;26:9:940216. https://doi.org/10.3389/fvets.2022.

61. Williamson JR, Callaway TR, Lourenco JM, Ryman VE. Characterization of rumen, fecal, and milk microbiota in lactating dairy cows. Front Microbiol. 2022;13:984119. https://doi.org/10.3389/fmicb.2022.984119.

62. Ruvalcaba-Gómez JM, et al. Growth performance and fecal microbiota of dairy calves supplemented with autochthonous lactic acid Bacteria as Probiotics in Mexican Western family dairy farming. Anim (Basel). 2023;13(18):2841. https://doi.org/10.3390/ani13182841.

63. Forno-Bell N, Bucarey SA, García D, Iragüen D, Chacón O, San Martín B. Antimicrobial effects caused by Aloe barbadensis Miller on bacteria associated with mastitis in dairy cattle. Nat Prod Commun. 2019;14:1934578X1989667. https://doi.org/10.1177/1934578X19896670.

64. Maan AA, Nazir A, Khan MKI, Ahmad T, Zia R, Murid M, Abrar M. The therapeutic properties and applications of Aloe: A review. J Herb Med. 2018;12:1–10. https://doi.org/10.1016/j.hermed.2018.01.002.

65. Pol M, Ruegg PL. Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J Dairy Sci. 2007;90(1):249–61. https://doi.org/10.3168/jds.S0022-0302(07)72626-7.

66. Serna-Cock L, Pabón-Rodríguez OV. Development of a teat bio-sealant and evaluation of its Technological and Functional properties. Probiotics Antimicrob Proteins. 2016;8(2):111–9. https://doi.org/10.1007/s12602-016-9210-5.

67. Trevisi E, Grossi P, Cogrossi S, Piccioli-Cappelli F. Effetti della somministrazione di Aloe Nel Periparto Sulla qualità Del latte bovino. Sci Tecn Latt -Cas. 2013;64(3–4):133–42.

68. Mezzetti M, Minuti A, Bionaz M, Piccioli-Cappelli F, Trevisi E. Effects of Aloe arborescens whole plant homogenate on lipid metabolism, inflammatory conditions and liver function of dairy cows during the transition period. Animals. 2020;10:917. https://doi.org/10.3390/ani10050917.

69. Zhu H, Miao R, Tao X, Wu J, Liu L, Qu J, Liu H, Sun Y, Li L, Qu Y. Longitudinal changes in milk microorganisms in the first two months of Lactation of Primiparous and Multiparous Cows. Anim (Basel). 2023;13(12):1923. https://doi.org/10.3390/ani13121923.

70. Addis MF, Tanca A, Uzzau S, Oikonomou G, Bicalho RC, Moroni P. The bovine milk microbiota: insights and perspectives from -omics studies. Mol Biosyst. 2016;12(8):2359–72. https://doi.org/10.1039/c6mb00217j.

71. Oikonomou G, Bicalho ML, Meira E, Rossi RE, Foditsch C, Machado VS, Teixeira AG, Santisteban C, Schukken YH, Bicalho RC. Microbiota of cow's milk; distinguishing healthy, sub-clinically and clinically diseased quarters. PLoS ONE. 2014;9(1):e85904. https://doi.org/10.1371/journal.pone.0085904.

72. Jiménez E, de Andrés J, Manrique M, Pareja-Tobes P, Tobes R, Martínez-Blanch JF, Codoñer FM, Ramón D, Fernández L, Rodríguez JM. Metagenomic Analysis

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of Milk of Healthy and mastitis-suffering women. J Hum Lact. 2015;31(3):406–15. https://doi.org/10.1177/0890334415585078.

73. Carson DA, Barkema HW, Naushad S, De Buck J. Bacteriocins of non-aureus Staphylococci isolated from Bovine Milk. Appl Environ Microbiol. 2017;83(17):e01015–17. https://doi.org/10.1128/AEM.01015-17.

74. Cerdó T, Ruiz A, Acuña I, Jáuregui R, Jehmlich N, Haange SB, von Bergen M, Suárez A, Campoy C. Gut microbial functional maturation and succession during human early life. Environ Microbiol. 2018;20(6):2160–77. https://doi.org/10.1111/1462-2920.14235.

75. Nogacka AM, Salazar N, Arboleya S, Suárez M, Fernández N, Solís G, de Reyes-Gavilán L, Gueimonde CG. Early microbiota, antibiotics and health. Cell Mol Life Sci. 2018;75(1):83–91. https://doi.org/10.1007/s00018-017-2670-2.

76. Mtshali K, Khumalo ZTH, Kwenda S, Arshad I, Thekisoe OMM. Exploration and comparison of bacterial communities present in bovine faeces, milk and blood using 16S rRNA metagenomic sequencing. PLoS ONE. 2022;17(8):e0273799. https://doi.org/10.1371/journal.pone.0273799.

77. Biscarini F, Cremonesi P, Castiglioni B, Stella A, Bronzo V, Locatelli C, Moroni P. A randomized controlled trial of teat-sealant and Antibiotic Dry-Cow Treatments for Mastitis Prevention Shows Similar Effect on the Healthy Milk Microbiome. Front Vet Sci. 2020;7:581. https://doi.org/10.3389/fvets.2020.00581.

78. Bonsaglia ECR, Gomes MS, Canisso IF, Zhou Z, Lima SF, Rall VLM, Oikonomou G, Bicalho RC, Lima FS. Milk microbiome and bacterial load following dry cow therapy without antibiotics in dairy cows with healthy mammary gland. Sci Rep. 2017;7(1):8067. https://doi.org/10.1038/s41598-017-08790-5.

79. Vlaeminck B, Fievez V, Cabrita ARJ, Fonseca AJM, Dewhurst RJ. Factors affecting odd- and branched-chain fatty acids in milk: a review. Anim Feed Sci Technol. 2006a;131:389–417.

80. Buccioni A, Decandia M, Minieri S, Molle G, Cabiddu A. Lipid metabolism in the rumen: new insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. Anim Feed Sci Technol. 2012; 1741–25.

81. Conte G, Palombo V, Serra A, Correddu F, D'Andrea M, Macciotta NPP, Mele M. Study of the Fatty Acid Profile of Milk in different Sheep breeds: evaluation by Multivariate Factorial Analysis. Animals. 2022;12:722. https://doi.org/10.3390/ani12060722.

82. Mele M, Conte G, Castiglioni B, Chessa S, Macciotta NPP, Serra A, Buccioni A, Pagnacco G, Secchiari P. Stearoyl-coenzyme A desaturase gene polymorphism and milk fatty acid composition in Italian holsteins. J Dairy Sci. 2007;90:4458–65. https://doi.org/10.3168/jds.2006-617.