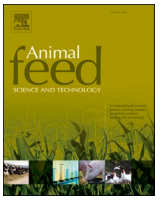
The impact of probiotic live yeast in a barley grain-based diet on rumen microbial communities, fermentation, and histology of artificially reared lambs

Animal Feed Science and Technology 322 (2025) 116269

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



The impact of probiotic live yeast in a barley grain-based diet on rumen microbial communities, fermentation, and histology of artificially reared lambs

Alexandros Mavrommatis [a], Marco Severgnini [b], Paola Cremonesi [c], Panagiota Kyriakaki [a], Christos Christodoulou [a], Konstantinos Petropoulos [d], Lysiane Dunière [e], Basiliki Kotsampasi [f], Bianca Castiglioni [c], Christos Balaskas [d], Eric Chevaux [e], Eleni Tsiplakou [a,*]

[a] Laboratory of Nutritional Physiology and Feeding, Department of Animal Science, School of Animal Biosciences, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[b] Institute of Biomedical Technologies – National Research Council (ITB-CNR), via f.lli Cervi 93, Segrate 20054, Italy
[c] Institute of Agricultural Biology and Biotechnology (IBBA-CNR), Via Einstein, Lodi 26900, Italy
[d] Laboratory of Anatomy & Physiology of Farm Animals, Department of Animal Science, School of Animal Biosciences, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
[e] Lallemand SAS, Blagnac 31702, France
[f] Research Institute of Animal Science, ELGO DIMITRA, Paralimni, Giannitsa 58100, Greece

ARTICLE INFO

Keywords:
Microbiome
Histology
Volatile fatty acids
Enzymatic activities
Gastrointestinal tract
Saccharomyces cerevisiae
Starch

ABSTRACT

This study aimed to investigate the effect of dietary supplementation with a probiotic live yeast *Saccharomyces cerevisiae* on rumen microbial communities, enzymatic activities, volatile fatty acid concentrations, and histology in artificially reared lambs fed a diet rich in fermentable carbohydrates. After colostrum administration, forty-two Chios lambs were divided into two homogenous groups ($n = 21$); the control (C) and probiotic supplemented group (P) and were artificially reared until the 45th day (d) of age. From the 30th until the 106th d of lambs' age, both groups were fed alfalfa hay and barley grain-based concentrate mix *ad libitum*. Moreover, 100 g of concentrate including 0.1 g of *Saccharomyces cerevisiae* CNCM I-1077 live yeast (10^{10} CFU/g) was offered to P lambs to ensure a constant intake of the yeast. Although no difference was evident for the alpha-diversity, several interesting features were found for beta-diversity. *Butyrivibrio, Pseudobutyrivibrio,* and acetate producer *Sphaerochaeta* were significantly more abundant ($P = 0.029$, $P = 0.029$ and $P = 0.008$, respectively) in P- compared to C-fed lambs at 100 d, while population of lactate users were varying significantly in P- and C- fed lambs at 100 d [higher abundance of *Megasphaera* in C- ($P = 0.001$) and *Anaerovibrio* in P-fed lambs ($P = 0.046$), respectively]. *Acidaminococcaceae* and *Clostridia UCG-014* were found in a lower abundance ($P = 0.059$ and $P = 0.008$, respectively) in P- compared to C-fed lambs at 100 d. Both ruminal pH and the activities of fibrolytic enzymes did not differ amongst the dietary treatments, while total volatile fatty acid concentrations were increased ($P < 0.001$) in P- compared to C-fed lambs at 100 d. As

Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; CFU, colony-forming unit; C, control; CP, crude protein; DM, dry matter; EE, ether extract; E, end of experimental period; LY, live yeasts; NDF, neutral detergent fiber; NFC, non- fiber carbohydrates; SARA, subacute ruminal acidosis; P, Probiotic supplementation; VFA, volatile fatty acids; W, weaning age.

* Corresponding author.
E-mail address: eltsiplakou@aua.gr (E. Tsiplakou).

https://doi.org/10.1016/j.anifeedsci.2025.116269
Received 22 November 2024; Received in revised form 9 February 2025; Accepted 11 February 2025
Available online 14 February 2025

regards the serosa, the muscular layers, the intramural vasculature and innervation, our results showed no effect in rumen histology due to the dietary treatment, however, changes were evident in the mucosa where all constituent layers of the epithelium presented distinctly and with similar thickness, especially around papillae, the collagen and reticular fibers were more densely and evenly packed, and prekeratins and keratins delineated more distinctly epithelial pegs in P-fed when compared to C-fed lambs. The inclusion of live yeast *S. cerevisiae* CNCM I-1077 promoted rumen microbiota stability and limited extensive keratinization of the epithelium due to a highly fermentable carbohydrate diet formulation.

1. Introduction

On commercial dairy sheep farms, lambs are frequently separated from their mothers and raised on milk replacers, which allows farmers to maximize milk production and revenue. However, artificial rearing can expose animals to digestive disorders and reconfigure their gastrointestinal tract microbial communities into a dysbiotic model during their early life (Chaucheyras-Durand et al., 2019; Arshad et al., 2021). Besides, the transition of stall-fed lambs from milk into solid feed is based on concentrate and consequently high starch content, which is rapidly fermented in the rumen and increases the risk of metabolic disorders (Commun et al., 2009). This condition occurs mainly due to an excessive accumulation of organic acids such as lactic acid in the rumen, which causes imbalances on ruminal pH (Oetzel, 2003). Ruminal acidity leads also to the lysis of gram-negative bacteria with the corresponding release of endotoxins, activating a cascade of inflammatory mediators, and affecting the animal's performance (Garcia Diaz et al., 2018). In a previous study, the increase of starch fermentability in the rumen of sheep through the proportional replacement of corn by barley grains resulted in a lower abundance of Bacteroides, *Butyrivibrio, Pseudobutyrivibrio, Ruminococcus,* and *Treponema* and a lower pH (Zhang et al., 2022). Moreover, Firmicutes, a phylum consisting mainly of species with fibrolytic activity were also decreased (Zhang et al., 2022). Previous studies have also reported that although the early life nutritional intervention has shaped the initial rumen microbial colonization and animal performance until weaning, the persistence of these effects later in life was weak (Belanche et al., 2019). Instead, the histological footprint of such stressors can be permanent on rumen epithelium. Several studies conducted on cows and goats have demonstrated that diets rich in grains or those inducing subacute ruminal acidosis (SARA) can disrupt the rumen epithelial barrier (Wang et al., 2021). This disruption is evidenced by morphological and histological damages to the ruminal papillae, increased lesions, and heightened paracellular permeability of the ruminal epithelium (Zhang et al., 2017; Zhao et al., 2018). Additionally, parakeratosis of the ruminal papillae, caused by the accumulation of keratinized, nucleated squamous epithelial cells and excessive epithelial sloughing, can hinder the absorption of volatile fatty acids (VFAs), thereby compromising the host's energy metabolism (Steele et al., 2009).

To overcome these gastrointestinal challenges, live yeasts (LYs), such as *Saccharomyces cerevisiae*, have commonly been used to improve digestive performance in young ruminants. In dam-reared lambs, LYs enhance the microbial colonization process of the rumen, while in gnotobiotically-reared lambs reared under sterile conditions, they stimulate the proliferation of fibrolytic bacteria (Chaucheyras-Durand and Fonty, 2001; Chaucheyras-Durand and Fonty, 2002; Fonty and Chaucheyras-Durand, 2006). Moreover, LYs act upon oxygen scavengers in rumen, enhancing anaerobiosis and activities of bacterial degradation (Fonty and Chaucheyras-Durand, 2006). Besides, LYs have the potential to support the digestive tract microbiota, ruminal pH regulation, and immunostimulation improving digestibility, performance, and health of animals. Additionally, LYs can either compete with lactic acid-producing bacteria like *Streptococcus bovis* and *Lactobacillus* for fermentable carbohydrates or support the growth of lactate-utilizing bacteria, which could help reduce lactate accumulation and maintain a higher ruminal pH (Nisbet and Martin, 1991; Chaucheyras et al., 1996; Chaucheyras-Durand and Fonty, 2001, 2006; Bach et al., 2007). Maintaining optimum ruminal conditions during nutritional challenges can not only balance biochemical functions but also prevent rumen epithelium from damage.

To address the challenges outlined, this study examined the effects of a probiotic LY supplement on rumen microbial structure, enzymatic activity, VFA concentrations, and histology in lambs subjected to two common stressors—artificial rearing and a diet high in fermentable carbohydrates—from early life through the fattening period. To simulate these stress conditions, we formulated a concentrate mix based on barley grains, in which starch is more fermentable and degrades more rapidly compared to corn (DelCurto-Wyffels et al., 2021).

2. Material and methods

2.1. Animals and housing conditions

This study extends previous research that examined the effects of live yeast supplementation on lamb performance and carcass traits. The experimental design was initially presented by Mavrommatis et al. (2024). Briefly, forty-two male Chios lambs were allocated into two homogenous groups ($n = 21$): a) control (C) and b) probiotic LY supplementation (P) and were artificially reared until 45 days after birth (weaning age; W). Throughout the rearing, the consumption of milk replacer was daily recorded on an individual basis. From the 30th until the 106th day of lambs' age, both groups (C and P) were fed *ad libitum* alfalfa hay, and the C-concentrate mix in individual pens. Moreover, 100 g of P-concentrate which included 0.1 g of *Saccharomyces cerevisiae* CNCM I-1077 live yeast (10^{10} CFU/g of commercial product) was offered to P-fed lambs. The latter sub-meal was provided before the feeding only in

the P group to ensure that lambs would receive a constant amount of the LY. The diets were designed using the nutritional dynamic system (NDS) (version 3.9.10.a). The two concentrate mixes (C and P) based on barley grain were formulated and produced in pellet form, while non-chopped alfalfa hay was separately provided as forage. Yeasts were enumerated after pelleting and over the experimental period (1.1×10^7 CFU/g feed) and were in line with the target concentration (1.0×10^7 CFU/g feed). Thus, the exact supplementation level was therefore set at 1.1×10^9 CFU/lamb/day. The composition of the concentrate mixes and the chemical composition of the feeds are presented in Table 1. Feed analyses, yeast enumeration after pelleting, and further information about the experimental design are available by Mavrommatis et al. (2024).

2.2. Sampling procedure

On the 45^{th} and 100^{th} day of lambs' age (or 15^{th} and 70^{th} day of probiotic yeast supplementation) ten out of twenty-one lambs/group were randomly selected for rumen digesta collection. Lambs' feeding schedule was modified from the previous day aiming to collect the rumen content from all experimental lambs at 4 hours after the morning feeding. Rumen digesta was collected using an electric vacuum pump at 2 mbar (VSV10, UNITAIR, Greece) and a stomach tube (flexible PVC tube of 1.5 mm thickness and 8 mm I.D.) as has been described for goats in early life by Belanche et al. (2020) and considering the protocols of Muizelaar et al. (2020). The stomach tube was placed at a depth up to 40–60 cm, while the first quantity of fluid (approx. 20–30 mL) was discarded to reduce the effect of the saliva contamination. Rumen digesta was collected in pre-warmed 1 L glass bottles, transferred into 50-mL plastic tubes (two aliquots), and pH was measured before they were snap-frozen in liquid nitrogen and stored at −80 °C until the analyses. In addition, on the 106th day of lambs' age, rumen wall portions dorsal to the cranial groove (from serosa to mucosa) were collected from all experimental lambs at the slaughterhouse, snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. Histology analyses

2.3.1. Fixation

Five out of twenty-one (per dietary group) rumen samples were randomly selected, slowly defrosted, and were subsequently immersed in 4 % paraformaldehyde (P6148 Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4, 79,382 Fluka, Buchs, Switzerland), at a volume of at least 20 times the volume of the tissue, for 1 h at room temperature. Tissues were washed two times for 30 min in PBS before storage overnight at 4 °C in PBS containing 7 % sucrose and 0.1 % sodium azide.

2.3.2. Cryostat sections

The fixed tissue was attached to a piece of cork (1 ×1 cm) and embedded in cryoprotection medium (Tissue-Tek, Jung), and rapidly frozen in liquid-nitrogen-cooled isopentane (AC126470010 Acros, Thermo Fisher Scientific, Waltham, MA, USA). Isopentane acts as a cryoprotective agent against liquid nitrogen. Four μm thick transverse sections were cut using Leica CM1500 (Leica Biosystems, Nussloch, Germany) cryostat were thaw-mounted on poly-L-lysine coated glass slides and finally stored at −4 °C. Cryotome sections relate more to fresh tissue compared to paraffin-embedded microtome sections due to the shrinkage caused by paraffin embedding, where tissues are exposed to temperatures of 40–60 °C, although the latter allows for easier sectioning and better quality for histology

Table 1
Concentrate ingredients and chemical composition of the feeds.

Item[a]	C-concentrate	P-concentrate		
Ingredients (g/kg, as fed)				
Crushed maize	184.0	183.0		
Soybean meal (44 % CP[b])	251.3	251.3		
Barley	549.2	549.2		
Mineral and vitamin premix	15.5	15.5		
Probiotic yeast product[c]	-	1		
Chemical composition (g/kg dry matter except as noted)	C-concentrate	P-concentrate	Alfalfa hay	Milk powder
Dry matter (as fed)	908 ± 1.2	905 ± 2.6	913 ± 0.8	953 ± 2.9
Ash	54 ± 1.5	58 ± 2.4	107 ± 0.7	72 ± 1.1
Crude protein	194 ± 4.5	192 ± 4.6	244 ± 4.0	230 ± 3.5
Ether extract	24 ± 1.2	27 ± 0.8	14 ± 0.8	230 ± 0.7
Non-fiber carbohydrates[d]	737 ± 3.9	750 ± 6.1	637 ± 4.6	-
aNDF[e]	212 ± 9.0	201 ± 18.6	442 ± 31.9	-
ADF[f]	71 ± 4.7	71 ± 9.6	303 ± 24.2	-
ADL[g]	20 ± 7.6	26 ± 6.8	72 ± 3.7	-
Starch	432 ± 21.9	444 ± 10.6	-	-

[a] Means ± standard deviation; SD from six samples per feed.
[b] CP = Crude protein.
[c] *Saccharomyces cerevisiae* CNCM I-1077 yeast as Levucell SC Titan (10e10 cfu/g).
[d] Non-fiber carbohydrates = 100 %-(Crude Protein% - Ether extract% - aNDF% - Ash%).
[e] aNDF = Neutral detergent fiber.
[f] ADF = Acid detergent fiber.
[g] ADL = Acid detergent lignin.

observations. However, cryotome sections in this study bear an additional advantage: rumen mucosa is associated with microflora which would be easily extracted in the process of paraffin embedding.

2.3.3. Microscopy slides poly-L-lysine coating

Untreated slides were washed in acid alcohol solution (70 % ethanol and 1 % HCl 1 N) for 90 min followed by running tap water for additional 90 min. After the washing, slides were dipped into poly-L-lysine solution (P8920 Sigma-Aldrich, St. Louis, MO, USA) for 10 sec. Finally, they were drained in the oven (37 °C) for 24 h.

2.3.4. Histochemistry

The sections were subsequently processed for histochemistry and treated with the following 4 histochemical stains, to assess at a preliminary level basic characteristics of the rumen histology: a) Haematoxylin and eosin to display tissue morphology, b) Mallory's trichrome according to McFarlane to display collagen, elastic fibers, and smooth muscle, c) Modified reticular fibers stain to display reticular fibers, and d) Dane's stain to display prekeratins, keratins, and mucins.

2.3.5. Microscopy

Stained sections were examined under a light microscope OLYMPUS BX50 fitted with image analysis Image Pro Plus V3.0.1 for Windows 7 HOME PREMIUM (Media cybernetics, Rockville, MD, USA).

2.4. DNA extraction and metagenomic analysis

DNA extraction was performed using a modified protocol combining enzymatic (Proteinase K), chemical (cetyl-trimethylammonium bromide; CTAB), and mechanical (liquid nitrogen using mortar and pestle) lysis properties as described by Mavrommatis et al. (2021a). Extensive quality assessment with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) were performed and eight (n = 8) samples per dietary group per sampling date were selected based on their quality assessment results for the subsequent library. Bacterial DNA was amplified using primers described in the literature (Caporaso et al., 2011) which target the V3-V4 hypervariable regions of the 16S rRNA gene. All PCR amplifications were performed in 25-μL volumes per sample. A total of 12.5 μL Phusion high-fidelity master mix 2 × (Thermo Fisher Scientific, Waltham, MA, USA) and 0.2 μL of each primer (100 μM) was added to 2 μL genomic DNA as template (5 ng/μL). Blank controls (i.e., no DNA template added to the reaction) were also included. A first amplification step was performed in an Applied Biosystems 2700 thermal cycler (Thermo Fisher Scientific). The samples were denatured at 98 °C for 30 sec, followed by 25 cycles with a denaturing step at 98 °C for 30 sec, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. The amplicons were cleaned using Agencourt AMPure XP beads (Beckman, Coulter, Brea, CA, USA), and libraries were prepared

Table 2
Oligonucleotide primers of selected targets using quantitative real time PCR.

Target	Region	Sequence (5'-3')		°C	bp	References
Total bacteria	16 s rRNA	Forward	CGGCAACGAGCGCAACCC	60.0	130	Denman and McSweeney (2006)
		Reverse	CCATTGTAGCACGTGTGTAGCC			
Bacteroidetes	16 s rRNA	Forward	GGARCATGTGGTTTAATTCGATGAT	62.0	126	Bahl et al. (2012)
		Reverse	AGCTGACGACAACCATGCAG			
Firmicutes	16 s rRNA	Forward	GGAGYATGTGGTTTAATTCGAAGCA	62.0	126	Bahl et al. (2012)
		Reverse	AGCTGACGACAACCATGCAC			
Neocallimastigales	18 s rRNA-ITS1	Forward	TTGACAATGGATCTCTTGGTTCTC	63.0	110	Kim et al. (2017)
		Reverse	GTGCAATATGCGTTCGAAGATT			
Saccharomyces cerevisiae	D1/D2	Forward	AGGAGTGCGGTTCTTTG	56.0	310	Chang et al., (2007)
		Reverse	TACTTACCGAGGCAAGCTACA			
Protozoa	18 s rRNA	Forward	GCTTTCGWTGGTAGTGTATT	55.0	223	Sylvester et al., (2004)
		Reverse	CTTGCCCTCYAATCGTWCT			
Prevotella sp.	16 s rRNA	Forward	GGTTCTGAGAGGAAGGTCCCC	60.0	121	Kim et al. (2017)
		Reverse	TCCTGCACGCTACTTGGCTG			
Butyrivibrio fibrisolvens	16 s rRNA	Forward	TAACATGAGAGTTTGATCCTGGCTC	58.0	136	Yang et al. (2009)
		Reverse	CGTTACTCACCCGTCCGC			
Ruminococcus flavefaciens	16 s rRNA	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	60.0	132	Denman and McSweeney, (2006)
		Reverse	CGGTCTCTGTATGTTATGAGGTATTACC			
Ruminococcus albus	16 s rRNA	Forward	CCCTAAAAGCAGTCTTAGTTCG	62.0	175	Kim et al. (2017)
		Reverse	CCTCCTTGCGGTTAGAACA			
Streptococcus bovis	16 s rRNA	Forward	TTCCTAGAGATAGGAAGTTTCTTCGG	57.0	127	Elolimy et al. (2018)
		Reverse	ATGATGGCAACTAACAATAGGGGT			
Fibrobacter succinogenes	16 s rRNA	Forward	GGCGGGATTGAATGTACCTTGAGA	60.0	204	Yang et al. (2009)
		Reverse	TCCGCCTGCCCCTGAACTATC			
Bifidobacterium	16 s rRNA	Forward	GCGTGCTTAACACATGCAAGTC	60.5	126	Pedersen et al. (2018)
		Reverse	CACCCGTTTCCAGGAGCTATT			
Lactobacillus	16 s rRNA	Forward	AGCAGTAGGGAATCTTCC	54.0	341	Pedersen et al. (2018)
		Reverse	ACACCGCTACACATGGAG			

following the 16S Metagenomic Sequencing Library Preparation Protocol (Illumina, San Diego, CA, USA). One sample was lost during library preparation and had no sequences. The libraries obtained were quantified by real-time PCR using KAPA library quantification kits (Kapa Biosystems, Inc., MA, USA), pooled in equimolar proportions, and sequenced in one MiSeq (Illumina) run with 2 × 250-bp paired-end reads. The raw 16S rRNA sequences were processed through a pipeline, including fragment rebuilding by PANDAseq (Masella et al., 2012) and quality filtering aimed at removing low-quality reads (i.e., showing stretches of bases with a Q score of <3 for more than 25 % of their length). Bioinformatic analyses were conducted using the QIIME pipeline release 1.9.0 suite (Caporaso et al., 2010), clustering filtered reads into operational taxonomic units (OTUs) at the 97 % identity level. In order to sort out putative chimeras, singleton OTUs (i.e., supported by fewer than 5 reads across all samples) were removed (Edgar, 2016). Taxonomic assignment was performed by the RDP classifier (Wang et al., 2007) against SILVA 138 database (Quast et al., 2013) using 0.8 as the confidence threshold. The data set was downsampled to the least sequenced sample to have a comparable picture of the taxonomic composition.

2.5. Quantitative PCR analysis for selected rumen microorganism

The primers set used for the real-time qPCR, the genomic region of PCR amplification, the amplicon size, and the primers' hybridization temperature are listed in Table 2. Primers targeting the 16S rRNA gene for total bacteria, initially developed by Denman and McSweeney (2006), served as a reference. Relative abundance was calculated using the formula: efficiency (target) $^{-\text{(Ct target microorganism-Ct of bacteria)}}$, as described by Chen et al. (2008) and Carberry et al. (2012). Quantitative real-time PCRs were performed using a Step-One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a reaction volume of 10 μL: 5 μL SYBR™ Select Master Mix (Thermo Fisher Scientific, Massachusetts, USA), 4 μL primers (0.2 μmol each), and 1 μL of DNA (20 ng/μL) as a template according to master mix manufacturer protocol. Validation of PCR efficiency, formation of a single product, primers specificity, and primers dimers are described by Mavrommatis et al. (2021a).

2.6. Ruminal enzymatic activities and volatile fatty acid concentrations

Ten mL of the rumen digesta were centrifuged at 13,000 × g at 4 °C for 5 min and then the supernatant was filtered under natural pressure through a polytetrafluoroethylene (PTFE) 0.45 μm syringe filter (Macherey Nagel, Germany) and stored in four aliquots at −80 °C until the analysis of enzymatic activities. Each aliquot was defrosted only once to ensure enzyme functionality. Alpha-amylase and protease activities were measured using a UV/Vis spectrophotometer (GENESYS 180, Thermo Fisher Scientific, Waltham, MA, USA) as described by Mavrommatis et al. (2021b). Cellulase and xylanase activities were determined using the Petri dish method. Briefly, a medium containing 37 mM KH_2PO_4, 11 mM K_2HPO_4, 0.4 mM $MgSO_4{\cdot}7\ H_2O$, 7.6 mM $(NH_4)2SO_4$, 27 mM microcrystalline cellulose at pH 5.5, and 15 g/L agar (w/v) was used for cellulase activity. The same procedure was followed for xylanase activity by using 10 g/L xylan from corn core as substrate. After the inoculation of the rumen fluid, the dishes were incubated at 39 °C for 20 h and then the hydrolytic halo was visualized by spreading 5 mL of the iodine solution (Mavrommatis et al., 2021b). Standard curves were obtained by consecutive dilutions of endocellulase (Aspergillus niger; Megazyme Ltd., Wicklow, Ireland) and endo-1–4-beta-Xylanase M1 (Trichoderma viride; Megazyme Ltd., Wicklow, Ireland). The ImageJ densitometry software (version 1.6, National Institute of Health, Bethesda, MD, USA) was used for clearance zone quantitative analysis.

Rumen samples were also used to measure VFA concentrations. More specifically, 0.8 mL of rumen fluid supernatant from the previous centrifugation (without the filtration) was acidified with 0.2 mL of 25 % metaphosphoric acid. After 30 min of incubation at 4 °C, samples were centrifuged at 13,000 × g at 4 °C for 5 min and then the supernatant was diluted with cold extra pure water (1:2). Samples were then injected in an Agilent 6890 N gas chromatograph equipped with an HP-FFAP capillary column (30 m × 0.25 mm i.d. with 0.20 μm film thickness, Agilent, Santa Clara, CA, USA) and a flame ionization detector (FID). One μL of the sample was injected using a split ratio 20:1 at 220 °C with air flow 450 mL/min. Oven temperature set at 140 °C, then increased to 220° C, at a rate of 5 °C/min, and held for 1 min. Detector set at 240 °C while H_2 flow set at 25 mL/min. Helium used as make up gas. Each peak was identified and quantified using a Volatile Fatty Acids Mix (Supelco, Sigma-Aldrich, St. Louis, MO, USA).

2.7. Statistical analyses

The alpha diversity, which estimates the microbial species diversity on a single sample scale, was measured using the Chao1, Shannon's diversity, observed species, and Faith's phylogenetic diversity indexes. A non-parametric permutation-based *t*-test (equivalent to Mann-Whitney U-test), with 999 random permutations was used to assess difference in the alpha-diversity. Weighted and unweighted UniFrac distances (Lozupone et al., 2011) and principal coordinates analysis (PCoA) were used to represent the microbial community structure for beta-diversity, which measures the variation of microbial communities between samples (Whittaker, 1960). The "Adonis" test function (Permutational Multivariate Analysis of Variance Using Distance Matrices, using pseudo-F ratios) was the statistical test used to define whether there is a significant difference among the experimental groups. The statistical test used for the analysis of rumen microbiota community was the non-parametric Mann-Whitney U-test using the SPSS Statistics 20.0 (IBM). Independent-sample *t*-test was applied to ruminal pH, VFA, and enzymatic activities since these variables followed a normal distribution. Statistical significance was set at $p < 0.05$ while $0.05 \leq P < 0.10$ was considered as a tendency.

3. Results

3.1. Rumen microbial communities

The analysis of the rarefaction curves for both the chao1 and the observed species metrics determined that all the samples show a tendency toward reaching a plateau at around 10,000 reads. Two out of the 31 samples had < 5000 reads and were omitted from the dataset, keeping only those samples where we were confident in capturing the main components of the microbiota. All the downstream analyses were performed normalizing all samples to the least sequenced sample (n = 8.674). In the end, the dataset was made of 29 samples (7 C and 8 P at weaning; 45 d, and 8 C and 6 P at the end of the experiment; 100 d).

Considering together the effects of diet and time points on the rumen microbiota highlighted that, although no difference was evident for the alpha-diversity, several interesting features were found for the beta-diversity (Fig. 1), with microbial profiles statistically different for both metrics (P = 0.001 and P = 0.019 for the unweighted and weighted UniFrac distances, respectively; Supplementary table 1). Moreover, samples were different when comparing time points (45 d vs 100 d) within the same diet, both for C- (P = 0.002 and P = 0.01 for the unweighted and weighted UniFrac distances, respectively) and the P-fed lambs (P = 0.002, unweighted UniFrac distance only; Supplementary table 1). Further UniFrac distances and PCoA are available in Supplementary Fig 1, Fig. 2, and Fig. 3.

At the phylum level, Cyanobacteria tended to be less abundant (P = 0.081) in P- compared to C-fed lambs at 100 d (Table 3). Furthermore, their relative abundance was significantly higher (P < 0.001) in C-fed lambs and tended to be more abundant (P = 0.059) in P-fed lambs at 100 d compared to 45 d. Verrucomicrobiota were significantly less abundant (P = 0.004) in C-fed lambs at 100 d compared to 45 d. At the family level, *Ruminococcaceae* tended to be less abundant (P = 0.072) in C-fed lambs at 100 d compared to 45 d, while *Acidaminococcaceae* were significantly more abundant (P < 0.001) in C-fed lambs at 100 d compared to 45 d. Furthermore, *Acidaminococcaceae* and *Clostridia UCG-014* were found in a lower abundance (P = 0.059 and P = 0.008, respectively) in P- compared to C-fed lambs at 100 d. Interestingly, *Clostridia UCG-014* were also significantly increased (*P* = 0.004) in C-fed lambs from 45 d to 100 d. *Muribaculaceae* tended to be more abundant (P = 0.072) in C-fed lambs and was significantly more abundant in P-fed lambs (P = 0.013) at 100 d compared to 45 d. In contrast, *Bacteroidales F082* were significantly less abundant in both C- and P-fed lambs (P = 0.001 and P = 0.020, respectively) at 100 d compared to 45 d. Also, *Bacteroidales F082* tended to be more abundant (P = 0.081) in P- compared to C-fed lambs at 100 d. At genus level, *Prevotella* tended to decrease (P = 0.081) in P-fed lambs from 45 d to 100 d, while *Prevotella_7* and *Roseburia* were significantly increased (P = 0.014 and P < 0.001 respectively) only in C-fed lambs from 45 d to 100 d. *Succiniclasticum* were found significantly higher (P = 0.021) in C- compared to P-fed lambs at 45 d, while also a significant increase (P = 0.001) from 45 d to 100 d was found. *Prevotellaceae UCG-001* tended to be more abundant in P-fed lambs at both 45 d and 100 d compared (P = 0.094 and P = 0.059, respectively) to the C-fed lambs. *Butyrivibrio* were found significantly more abundant (P = 0.029) in P- compared to C-fed lambs at 100 d, while also their abundance was significantly decreased (P = 0.006) in C-fed lambs from 45 d to 100 d. *Acidaminococcus* significantly decreased (P = 0.029) in P- compared to C-fed lambs at 100 d, while also their abundance was significantly increased (*P* = 0.014) in C-fed lambs from 45 d to 100 d. *Anaerovibrio were* found significantly higher (P = 0.043) in P- compared to C- fed lambs at 100 d. *Pseudobutyrivibrio* and *Sphaerochaeta* were significantly more abundant (P = 0.029 and P = 0.008, respectively) in P- compared to C- fed lambs at 100 d, while also their abundances were significantly decreased (P = 0.002 and P < 0.001, respectively) in C-fed lambs from 45 d to 100 d. *Veillonellaceae UCG-001* was significantly more abundant

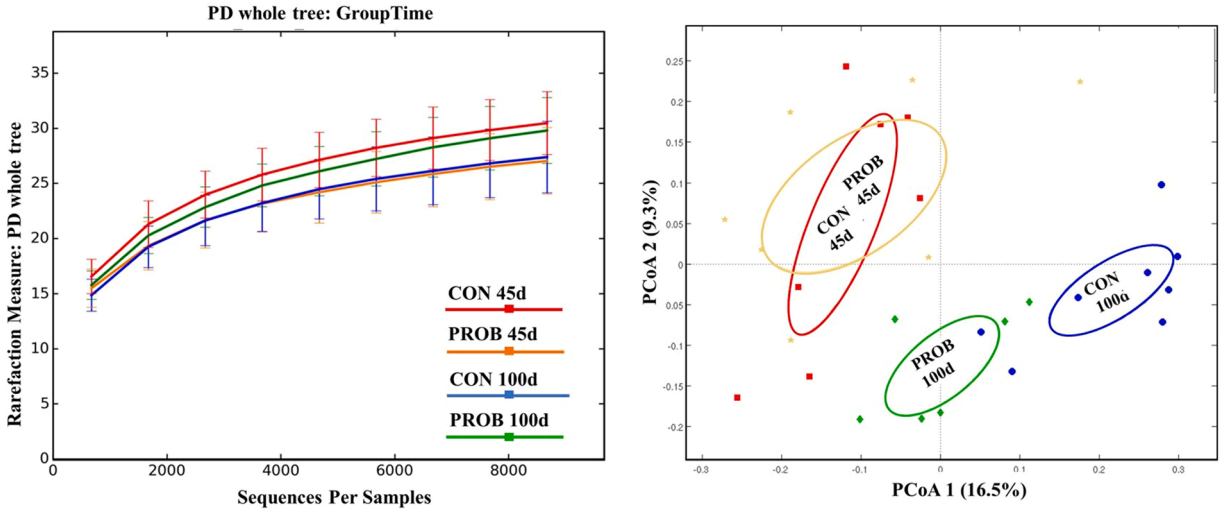


Fig. 1. A) Alpha diversity as PD whole tree and B) beta-diversity as Principal Coordinate Analysis (PCoA) of the two dietary treatments (C vs P) at two sampling times (W: 45th and E: 100th day). C = Control dietary treatment (*n* = 7 at W and n = 8 at E) and P = Probiotic dietary treatment supplemented with 1.1x10e9 CFU live yeast/day (*n* = 8 at W and n = 6 at E).

Table 3
Effect of probiotic yeast supplementation on relative abundance (%) of bacteria taxa in the rumen of (C and P) lambs at two sampling times (W: 45th and E: 100th day).

	Treatments				SEM	P value			
	W		E			W	E	C	P
	C	P	C	P		C *vs* P	C *vs* P	W *vs* E	W *vs* E
Phylum									
Bacteroidetes	44.20	44.04	40.03	43.11	1.034	0.955	0.282	0.121	1.000
Firmicutes	32.98	34.12	38.66	36.30	1.279	0.779	0.282	0.281	0.662
Proteobacteria	8.10	6.36	13.42	12.60	1.559	0.867	0.852	0.443	0.142
Spirochaetota	6.90	5.18	2.46	2.61	0.693	0.463	0.755	0.121	0.142
Fibrobacterota	2.72	4.34	0.70	1.22	0.514	0.416	0.228	0.152	0.282
Verrucomicrobiota	1.50	1.55	0.10	0.17	0.331	0.281	0.755	0.004	0.228
Cyanobacteria	0.13	0.17	1.99	0.93	0.199	0.536	0.081	< 0.001	0.059
Euryarchaeota	0.72	0.68	0.81	0.93	0.081	0.536	0.491	0.336	0.228
Family									
Prevotellaceae	31.96	33.40	31.28	34.43	1.093	0.681	0.491	0.779	0.852
Lachnospiraceae	10.93	9.87	14.20	14.51	0.894	0.918	1.000	0.232	0.142
Succinivibrionaceae	7.83	5.89	12.17	11.86	1.557	0.681	0.950	0.536	0.142
Spirochaetaceae	6.90	5.18	2.46	2.61	0.693	0.351	0.755	0.121	0.142
Ruminococcaceae	5.54	4.75	2.39	3.50	0.565	0.606	0.345	0.072	0.414
Acidaminococcaceae	2.59	2.00	6.86	3.19	0.530	0.142	0.059	< 0.001	0.573
Oscillospiraceae	4.18	4.70	2.24	2.63	0.539	1.000	0.662	0.152	0.142
Clostridia UCG−014	1.61	3.49	5.78	2.25	0.429	0.114	0.008	0.004	0.142
Rikenellaceae	4.71	4.55	1.82	2.42	0.584	0.681	0.282	0.094	0.282
Selenomonadaceae	2.92	2.28	1.62	6.87	0.767	0.758	0.142	1.000	0.228
Muribaculaceae	1.55	1.08	5.26	3.95	0.667	0.299	0.755	0.072	0.013
Bacteroidales F082	4.56	3.26	0.77	1.47	0.401	0.142	0.081	0.001	0.020
Fibrobacteraceae	2.72	4.34	0.70	1.22	0.514	0.606	0.228	0.152	0.282
Erysipelatoclostridiaceae	1.22	3.15	0.92	0.62	0.665	0.470	0.345	0.613	0.414
Kiritimatiellae (WCHB1–41)	1.32	1.45	0.10	0.16	0.310	0.252	0.755	0.004	0.414
Methanobacteriaceae	0.72	0.68	0.81	0.93	0.081	0.470	0.491	0.336	0.228
Bacteroidales RF16 group	0.91	1.08	0.49	0.31	0.230	1.000	0.414	0.955	0.491
Erysipelotrichaceae	0.55	0.71	0.89	0.44	0.106	0.408	0.414	0.397	0.662
Christensenellaceae	0.73	0.60	0.41	0.33	0.095	0.252	0.414	0.029	0.755
Genus									
Prevotella	24.70	24.51	20.14	18.81	1.246	1.000	0.755	0.536	0.081
Succinivibrio	5.06	4.01	11.74	8.87	1.573	0.779	0.662	0.336	0.181
Prevotella_7	0.83	1.84	7.69	4.57	1.316	0.613	0.142	0.014	0.181
Roseburia	0.44	0.66	8.40	5.64	0.986	0.779	0.282	< 0.001	0.081
Treponema	6.56	3.49	2.45	2.37	0.642	0.336	0.852	0.152	0.491
Clostridia UCG−014 (other)	1.61	3.49	5.78	2.25	0.429	0.054	0.008	0.004	0.142
Rikenellaceae RC9 gut group	4.53	4.54	1.80	2.42	0.586	0.779	0.282	0.121	0.282
Succiniclasticum	2.17	1.20	4.30	2.69	0.303	0.021	0.181	0.001	0.282
Prevotellaceae UCG−001	0.62	1.26	0.85	8.61	0.939	0.094	0.059	0.613	0.228
Fibrobacter	2.72	4.34	0.70	1.22	0.514	0.463	0.228	0.152	0.282
Ruminococcus	4.10	3.10	0.69	1.11	0.433	0.613	0.108	0.006	0.043
Bacteroidales F082	2.80	2.73	0.51	1.44	0.255	0.955	0.043	0.002	0.043
Unclassified Muribaculaceae	0.00	0.83	4.56	1.20	0.614	0.694	0.081	< 0.001	0.181
Selenomonas	1.62	0.00	0.89	5.03	0.676	0.694	0.142	0.121	< 0.001
Ruminobacter	2.41	1.59	0.18	2.60	0.601	0.463	0.142	0.397	0.345
Butyrivibrio	2.11	1.64	0.31	1.03	0.320	0.463	0.029	0.006	0.414
Acidaminococcus	0.42	0.79	2.56	0.50	0.301	0.867	0.029	0.014	0.755
Ruminococcaceae (other)	1.26	0.46	1.14	1.72	0.285	0.281	0.414	0.613	0.142
Anaerovibrio	0.68	1.19	0.53	0.97	0.159	0.613	0.043	0.463	0.852
Lachnospiraceae NK3A20 g	0.57	0.51	0.58	1.53	0.147	0.189	0.142	0.779	0.081
Prevotellaceae UCG−003	0.80	0.99	0.62	0.32	0.139	0.613	0.852	0.463	0.228
Pseudobutyrivibrio	1.37	0.80	0.12	0.47	0.180	0.189	0.029	0.002	0.662
Methanobrevibacter	0.58	0.62	0.70	0.82	0.070	0.867	0.414	0.397	0.228
Sphaerochaeta	0.33	1.68	0.01	0.25	0.202	0.336	0.008	< 0.001	0.142
F082 (other)	1.67	0.45	0.20	0.03	0.219	0.054	0.108	0.072	0.282
CAG−352	0.08	0.81	0.42	0.51	0.137	0.040	0.852	0.232	0.414
Lachnospira	0.48	0.76	0.16	0.29	0.128	0.955	0.491	0.694	0.852
Muribaculaceae (other)	0.69	0.04	0.56	0.40	0.161	0.121	1.000	0.536	0.081
Veillonellaceae UCG−001	0.37	0.46	0.09	0.79	0.126	0.779	0.013	0.336	0.228
Alloprevotella	0.52	0.81	0.06	0.11	0.117	0.536	1.000	0.029	0.029
Lachnospiraceae NK4A136 g	0.73	0.35	0.10	0.41	0.105	0.397	0.059	0.072	0.414
Oribacterium	0.20	0.37	0.05	0.99	0.181	0.336	0.228	0.054	0.491
Colidextribacter	0.83	0.48	0.06	0.02	0.131	0.867	0.228	0.536	0.142
Lachnoclostridium	0.14	0.06	0.63	0.58	0.126	0.397	0.662	0.867	0.108

(*continued on next page*)

Table 3 (*continued*)

	Treatments				SEM	P value			
	W		E			W	E	C	P
	C	P	C	P		C *vs* P	C *vs* P	W *vs* E	W *vs* E
RF39 (other)	0.67	0.34	0.18	0.08	0.140	0.463	0.282	0.536	0.228
Desulfovibrio	0.29	0.74	0.00	0.22	0.136	0.613	0.059	0.004	0.282
Megasphaera	0.10	0.13	0.90	0.05	0.095	0.281	0.001	0.009	0.662

C = Control dietary treatment ($n = 7$ at W and n = 8 at E) and P = Probiotic dietary treatment supplemented with 1.1x10e9 CFU live yeast/day ($n = 8$ at W and n = 6 at E).

(P = 0.013) in P- compared to C- fed lambs at 100 d. *Lachnospiraceae NK4A136 g* and *Desulfovibrio* tended to be more abundant (P = 0.059 and P = 0.059, respectively) in P- compared to C- fed lambs at 100 d. *Megasphaera* was found significantly less abundant (P = 0.001) in P- compared to C- fed lambs at 100 d since was significantly increased (P = 0.009) from 45 d to 100 d only in the C-fed lambs.

A real-time qPCR platform was also applied to validate sequencing results and further explore specific taxa other than prokaryotes. The main rumen phyla were confirmed to be not significantly affected by the treatment or the time variables (Table 4). On the other hand, qPCR data confirmed that *Prevotella* sp. was significantly higher (P < 0.001) in the C-fed lambs at 100 d compared to 45 d and *Ruminococcus* (in particular, *R. albus*) were found in a significantly lower relative abundance (P = 0.011) from 45 d to 100 d only in the C-fed lambs. Moreover, *Bifidobacterium* tended to be more abundant (P = 0.063) in the rumen of C- compared to P-fed lambs at 100 d, while *Lactobacillus* was significantly increased (P = 0.043) at 100 d compared to 45 d only in the C-fed lambs. Among fungi, *Saccharomyces cerevisiae* was found significantly higher (P < 0.001) in the rumen of P- compared to C-fed lambs at both 45 d and 100 d, whereas *Neocallimastigales* almost disappeared at 100 d in both C- and P-fed lambs (P = 0.021 and P = 0.040, respectively).

3.2. Rumen enzymatic activity and volatile fatty acid concentrations

Ruminal pH did not differ amongst dietary treatments while was found significantly lower at 100 d compared to 45 d for both C- and P- fed lambs (P = 0.005 and P < 0.001, respectively; Table 5). Amylase activity tended to increase (P = 0.055) in P- compared to C-fed lambs at 45 d, while at 100 d significantly higher activity was found in both C- and P- fed lambs (P = 0.001 and P = 0.010, respectively). Protease activity was found significantly higher (P = 0.014) in P- compared to C-fed lambs at 100 d. Cellulase activity was significantly higher (P < 0.001) in C-fed lambs at 100 d, while it tended to be higher (P = 0.056) in P-fed lambs at 100 d compared to 45 days. Similarly, xylanase activity was found significantly higher in both C- and P- fed lambs (P = 0.018 and P = 0.010, respectively) at 100 d compared to 45 d. Concentrations of total VFAs were found significantly increased (P < 0.001) in P- compared to C-fed lambs at 100 d, while tended to increase (P = 0.078) also at 45 d. Concentration of acetate (P < 0.001), propionate (P = 0.023), butyrate (P = 0.002), valerate (P = 0.021), and isobutyrate (P = 0.011) were significantly increased in P- compared to C-fed lambs at 100 d while only propionate was significantly increased (P = 0.041) at 45 d. On the contrary, molar proportions of VFAs were not significantly affected amongst the dietary treatments (Table 5).

Table 4
Effect of probiotic yeast supplementation on relative abundance (%) of selected microorganisms in the rumen of (C and P) lambs using RT-qPCR at two sampling times (W: 45th and E: 100th day).

	Treatments				SEM	P value			
	W		E			W	E	C	P
	C	P	C	P		C *vs* P	C *vs* P	W *vs* E	W *vs* E
Bacteroidetes	39.22	35.44	41.68	40.83	2.350	0.236	0.888	0.721	0.340
Firmicutes	35.14	32.49	35.5	33.36	3.001	0.963	0.673	0.743	0.888
Firmicutes/Bacteroidetes	0.91	1.01	1.07	1.09	0.146	0.673	0.370	0.442	0.489
Prevotella sp.	20.28	19.84	34.39	27.51	1.704	1.000	0.423	< 0.001	0.258
Butyrivibrio fibrisolvens	1.11	1.05	1.15	1.48	0.134	1.000	0.863	0.743	0.666
Ruminococcus flavefaciens, 10^{-2}	1.2	0.7	0.0	0.4	0.003	0.888	0.113	0.096	0.931
Ruminococcus albus, 10^{-1}	1.41	1.53	0.16	0.54	0.027	0.370	0.136	0.011	0.340
Streptococcus bovis, 10^{-2}	0.02	0.05	0.01	0.01	< 0.001	0.798	0.863	0.139	0.673
Fibrobacter succinogenes, 10^{-2}	0.2	1.2	0.03	0.15	0.001	0.613	0.666	0.046	0.091
Bifidobacterium, 10^{-1}	3.73	0.25	1.20	0.15	0.056	0.236	0.063	0.606	0.666
Lactobacillus10^{-3}	0.0	0.0	2.3	0.1	< 0.001	0.445	0.130	0.043	0.281
Neocallimastigales, *10^{-2}*	3.6	4.6	0.0	0.0	0.007	0.963	0.796	0.021	0.040
Saccharomyces cerevisiae 10^{-3}	0.0	0.9	0.0	0.7	< 0.001	< 0.001	< 0.001	0.541	0.222
Protozoa, *10^{-1}*	2.58	5.46	3.87	9.29	0.190	1.000	0.918	0.743	0.758

C = Control dietary treatment ($n = 7$ at W and n = 8 at E) and P = Probiotic dietary treatment supplemented with 1.1x10e9 CFU live yeast/day ($n = 8$ at W and n = 6 at E).

Table 5
Effect of probiotic yeast supplementation on ruminal pH, enzymatic activities, volatile fatty acids in lambs (C and P) at two sampling times (W: 45th and E: 100th day).

	Treatments				SEM	P value			
	W		E			W	E	C	P
	C	P	C	P		C *vs* P	C *vs* P	W *vs* E	W *vs* E
pH	7.20	7.12	6.69	6.34	0.098	0.539	0.115	0.005	< 0.001
Enzymatic activities									
Amylase, mg maltose released	5.74	7.80	9.68	11.39	0.698	0.055	0.201	0.001	0.010
Protease, Units/mL	0.12	0.15	0.11	0.16	0.020	0.264	0.014	0.869	0.865
Cellulase, Units/mL/h	40.63	45.21	54.33	53.97	7.356	0.182	0.927	< 0.001	0.056
Xylanase, Units/mL/h	16.83	17.58	24.96	23.05	2.156	0.654	0.579	0.018	0.010
VFA concentrations (mM)									
Total VFA	46.12	67.27	66.03	114.27	5.550	0.078	< 0.001	0.033	0.002
Acetate	31.40	43.56	32.05	55.71	2.591	0.086	< 0.001	0.898	0.091
Propionate	8.40	12.99	17.78	28.64	1.756	0.041	0.023	0.001	0.004
Butyrate	3.83	8.06	12.05	23.24	1.582	0.147	0.002	< 0.001	0.002
Valerate	0.55	0.63	1.66	2.37	0.149	0.532	0.021	< 0.001	< 0.001
Isobutyrate	1.04	1.14	1.84	3.50	0.226	0.609	0.011	0.048	0.001
Isovalerate	1.00	1.00	0.65	0.81	0.073	0.996	0.417	0.045	0.450
Acetate: Propionate	3.74	3.52	1.91	2.15	0.168	0.481	0.418	< 0.001	0.001
VFA molar proportions (mol/100 mol Total VFA)									
Acetate	68.32	66.10	48.43	49.07	1.702	0.226	0.792	< 0.001	< 0.001
Propionate	18.68	19.50	26.53	24.40	0.913	0.628	0.425	< 0.001	0.072
Butyrate	7.47	10.31	18.69	20.66	1.300	0.233	0.532	< 0.001	0.005
Valerate	1.17	0.97	2.70	2.11	0.151	0.154	0.150	< 0.001	0.001
Isobutyrate	2.38	1.80	2.62	2.99	0.153	0.132	0.411	0.591	0.004
Isovalerate	1.97	1.49	1.03	0.76	0.127	0.170	0.264	< 0.001	0.012

C = Control dietary treatment (n = 10 at W and n = 10 at E) and P = Probiotic dietary treatment supplemented with 1.1x10e9 CFU live yeast/day (n = 10 at W and n = 10 at E). Effect of probiotic yeast supplementation on ruminal volatile fatty acids in lambs (C and P) at two sampling times (W: 45th and E: 100th day).

3.3. Rumen histology

Our observations showed that the lambs' rumen wall consisted of four distinct layers: tunica adventitia (serosa), tunica muscularis (smooth muscle cell layer) comprising an outer longitudinal and inner circular layers and tunica mucosa. The latter (mucosa) was lined with keratinized stratified squamous epithelium consisting of five layers (stratum corneum, lucidum, granulosum, spinosum and basale) and beared leaf-like papillae either short (without lamina propria) or long (with lamina propria). Blood vessels and intramural ganglia and nerves were also evident in the rumen wall. As regards the serosa, the muscular layers, the intramural vasculature and innervation, our results showed no effect in rumen histology due to the dietary treatment. Differences, however, between C- and P-fed

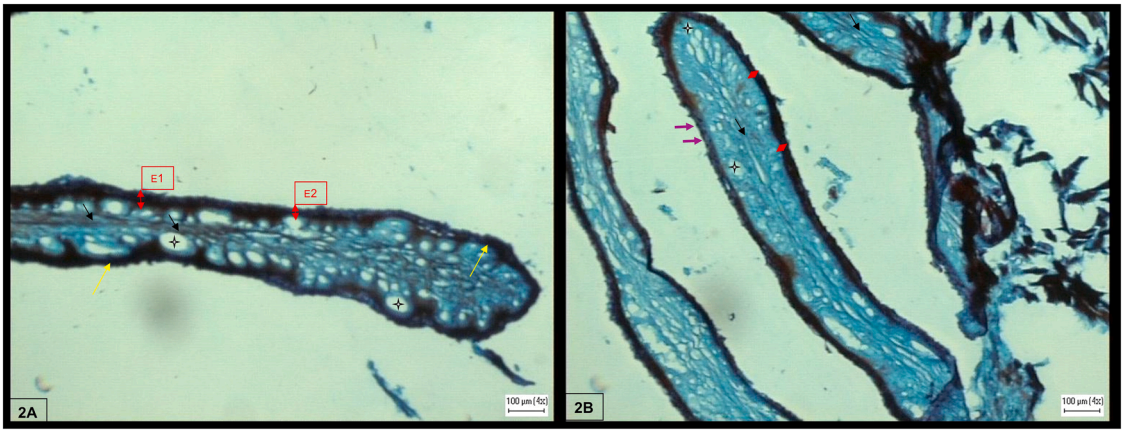


Fig. 2. Mallory's trichrome according to McFarlane stain on cryostat sections from rumen of C-fed (A) and P-fed lambs (B). The stain allowed for the visualization of collagen in the lamina propria (shades of blue), delineated blood vessels' lumens (stars) and labelled smooth muscle cells (arrows), but also showed that the epithelium was of variable thickness (red double arrows- compare E1 with E2) and its layers were not uniformly presented in the periphery of the papillae in control lambs, where keratinization (yellow arrows) was very prominent (Fig. 1A). In contrast, in P-fed lambs' papillae epithelium was of uniform thickness (Fig. 1B, compare red double arrows) and with well-delineated layers and often visible epithelial cell nuclei (purple arrows).

lambs' rumen histology were detected in the mucosa. These differences are depicted in Figs. 2, 3, and 4 (all using the same lens magnification). In both C- and P-fed lambs, the Mallory's trichrome according to McFarlane stain revealed the extensive presence of collagen in the lamina propria, delineated blood vessels' lumens, and labeled smooth muscle cells, but also showed that in C-fed lambs the epithelium was of variable thickness and its layers were not uniformly presented in the periphery of the papillae, where keratinization was very prominent (Fig. 2A). In contrast, in the P-fed lambs' papillae, epithelium was of uniform thickness and with well-delineated layers and often visible epithelial cell nuclei (Fig. 2B). The modified reticular fiber stain confirmed the presence of reticular and collagen fibers in the lamina propria (Fig. 3A) but confirmed that both (reticular and collagen fibers) were more densely packed and structurally supported, as they should, the mucosa and its constituents in P-fed lambs (Fig. 3B). As regards Dane's stain, the presence of prekeratins and keratins was evident in both C- and P-fed lambs' papillae (Fig. 4A and B), but their distribution was prominent in the P-fed lambs, delineating epithelial pegs, when compared to the C-fed lambs.

4. Discussion

In our study, lambs were artificially reared in good, well-controlled environmental and sanitary conditions, and growth parameters were comparable to the standard of the Chios breed of lambs. The performance, health indices, and carcass traits of lambs were previously reported in the first part of the present study (Mavrommatis et al., 2024).

Analysis of the microbiota profiles revealed that, although neither the dietary treatments nor the sampling time affected the biodiversity (alpha-diversity), the microbial profiles (beta-diversity) were significantly modified at 100 d as compared to 45 d for both dietary treatments and that at the end of the treatment (100 d), the probiotic diet led to a different bacterial composition than the control diet, whereas at 45 d no differences were recorded. Consistent with this observation, at family and genus levels, rumen microbiota communities showed alterations of several taxa mostly at 100 d. A plausible explanation for the lack of differences in rumen microbial structure between the C- and P-fed lambs at weaning may lie in the duration of probiotic yeast supplementation. At the time of the first sampling (45 d), the lambs had only received yeast supplementation for 15 d, whereas by the second sampling (100 d), they had been supplemented for 70 d. Given the limited solid feed intake during the early weeks of life, the effectiveness of probiotic supplementation might be influenced by the route of administration.

In our study, C-fed lambs presented a microbial profile more associated to animal under dysbiotic conditions, such as SARA, than the P-fed lambs with variations observed on *Prevotella*, lactate utilizers, fibrolytic and amylolytic populations. More specifically, *Prevotellaceae UCG-001* tended to be decreased in the C- compared to the P-fed lambs at 100 d, aligned with previous evidence where *Prevotellaceae UCG-001, Prevotellaceae UCG-003,* and *Prevotella 1* were significantly decreased during SARA using the rumen simulation technique (Brede et al., 2020). *Prevotella_7* (from metagenomic analysis) and *Prevotella sp.* (from RT-qPCR) were increased from 45 d to 100 d only in C-fed lambs, whereas *Clostridia UCG-014* were found more abundant at 100 d in the C-fed lambs rather than in the P-fed ones. In a previous study, ruminal pH was negatively correlated with *Prevotella, Syntrophococcus, Succinivibrionaceae UCG-001*, and *Clostridia UCG-014*. This suggests that at a higher pH, the relative abundance of these bacteria was lower (Ravelo et al., 2023). It should be highlighted that the different behavior of members of the *Prevotellaceae* species in our study confirms the metabolic divergence within their family, since in previous studies, different *Prevotella* species have been associated with both higher and lower feed efficiency in cattle and sheep (Ellison et al., 2017; Brooke et al., 2019; Delgado et al., 2019). For instance, Ellison et al. (2017) reported that the abundance of *Prevotella ruminicola* increased significantly for Low-RFI lambs when fed a concentrate-based diet and decreased in Low-RFI lambs when fed a forage-based diet. The opposite was reported for *Prevotella bryantii* (Ellison et al., 2017). In the same line with our results, *Ruminobacter amylophilus* was less abundant in SARA (Mickdam et al., 2016). *Acidaminococcus* increased in

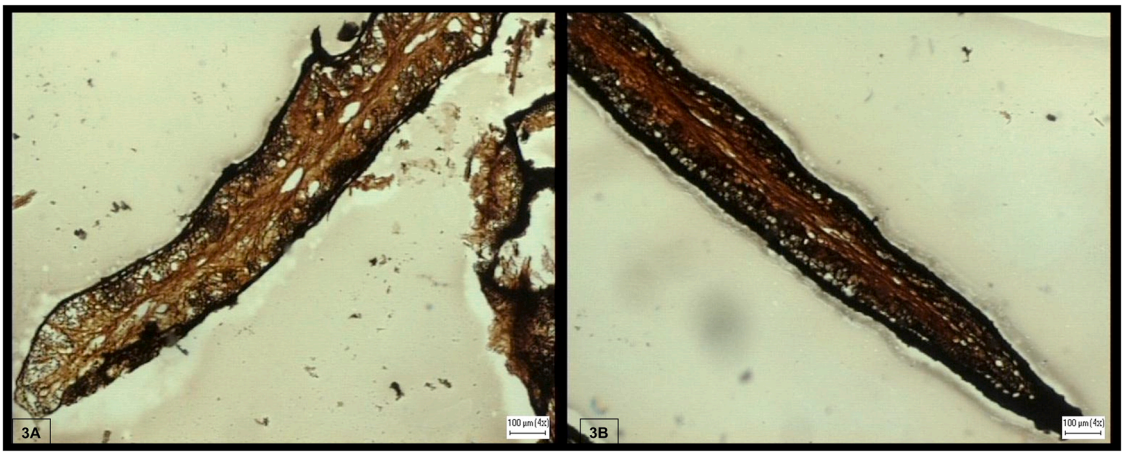


Fig. 3. Modified reticular fiber stain on cryostat sections from rumen of C-fed (A) and P-fed lambs (B). The stain showed the presence of reticular (black) and collagen fibers (brown) in the lamina propria but confirmed that both (reticular and collagen fibers) where more densely packed in the mucosa of P-fed lambs (compare A to B).

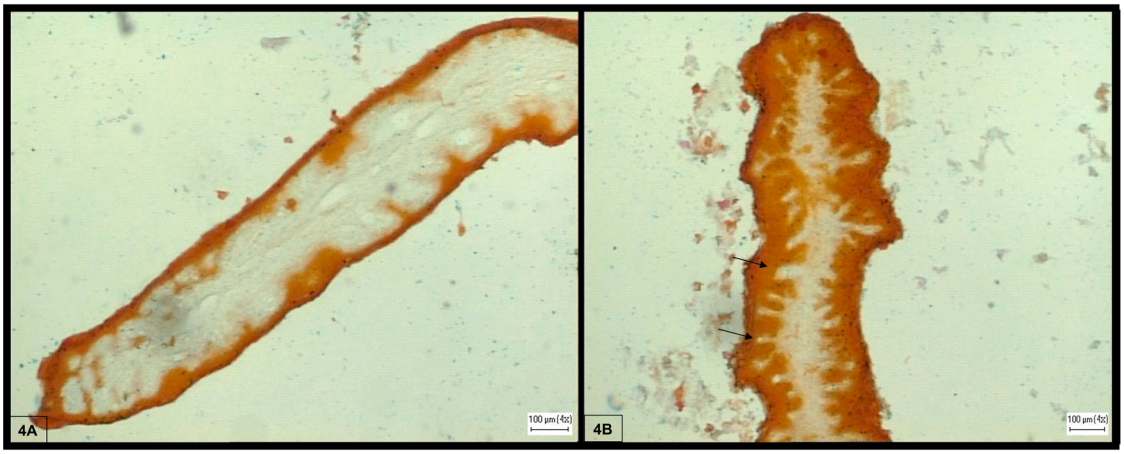


Fig. 4. Dane's stain on cryostat sections from rumen of C-fed (A) and P-fed lambs (B). The stain displayed the presence of prekeratins and keratins (shades of orange) in both C- and P-fed lambs' papillae. The distribution of both prekeratins and keratins was more prominent in the P-fed lambs, delineating epithelial pegs (black arrows), when compared to C-fed lambs.

the C- compared to the P-fed lambs at 100 d. In a previous study where heifers were exclusively fed forage, before being transitioned to a concentrate diet, and subjected to an acidotic challenge, *Acidaminococcus* increased in percent abundance as minimum pH decreased (Petri et al., 2013).

On the other hand, the lactate-utilizing bacterium *Anaerovibrio* and the fibrolytic bacterium *Butyrivibrio* exhibited greater abundance in P-fed lambs compared to C-fed lambs at 100 d. This increase could be linked to the influence of rapidly fermentable carbohydrates, which may inhibit the growth and activity of certain biohydrogenating bacteria (Fuentes et al., 2009). It is important to note that in our study, the relative abundance of *Butyrivibrio fibrisolvens*, as estimated through RT-qPCR, showed no significant differences between groups. However, other taxa, including *Pseudobutyrivibrio, Veillonellaceae UCG-001, Lachnospiraceae NK4A136 g*, and *Sphaerochaeta* were found to be more abundant in the P-fed lambs compared to the C-fed lambs at 100 d. This finding aligns with a previous study using a rumen simulation model for SARA, where *Butyrivibrio 2, Lachnospiraceae AC2004 group, Pseudobutyrivibrio, Schwartzia, Sphaerochaeta*, and *Veillonellaceae UCG-001* were reported to be less abundant in the solid phase of the rumen (Brede et al., 2020). Lastly, rumen microbiota analysis revealed that *Megasphaera* was found to increase in the C- compared to the P-fed lambs at 100 d. The abundance of *Megasphaera elsdenii* usually increases in the rumen of animals fed high-grain diets due to its ability to use lactate, especially at a low ruminal pH (Cabral and Weimer, 2024). *M. elsdenii* has been proposed as a potential dietary probiotic to prevent ruminal acidosis due to its potential to utilize lactate. The increase of *Megasphaera* up to 0.9 % of relative abundance in the rumen of C- compared to the P-fed lambs at 100 d and its possible utilization of lactate may have prevented a more severe impact of the barley grain diet in the lambs' performances (Oetzel, 2003). Other lactate utilizers were observed in numerical higher abundance in P- than in C-fed lambs at 100 d (*Selenomonas* at 5.03 % vs 0.89 % and *Anaerovibrio* at 0.97 % vs 0.53 % in P- and C- fed lambs, respectively), confirming an optimal rumen functioning regarding lactate metabolism in the probiotic group.

It has been reported that during ruminal dysbiotic conditions such as SARA, species belonging to *Fibrobacter* and the *Ruminococcaceae* and other important cellulolytic bacteria in the rumen (Berg Miller et al., 2009; Suen et al., 2011) tended to be decreased (Fernando et al., 2010; Petri et al., 2013) due to their sensitivity on pH alterations (Russell and Dombrowski, 1980). Nonetheless, *Ruminococcacceae* also contains members, that can ferment starch (Stewart et al., 1997) and, therefore, are sometimes reported to increase during high-grain diets (Khafipour et al., 2009; Mao et al., 2013). In a previous study, amylolytic bacteria, including *Prevotella, Streptococcus bovis, Selenomonas ruminantium,* and *Prevotella brevis* reported a linear increase with increasing dietary barley starch levels in diet of Hu sheep (Zhang et al., 2022). In our study, relative abundance of *Bifidobacterium* based on RT-qPCR, was higher in C- compared with P-fed lambs at 100 d. Ellison et al. (2017) found *Bifidobacterium,* the major lactic acid-producing bacteria, to be significantly more abundant in the rumen of lambs when fed a high-grain diet. Although it has been reported that *Bifidobacterium* is rapidly increased in diets based on concentrate, this genus, as well as *Prevotella*, were negatively correlated with the molar proportion of propionate in the rumen (Bi et al., 2018). In agreement with the previous observations, in our study, the concentration of propionate in the rumen depicted a negative correlation with both *Bifidobacterium and Prevotella.* Neocallimastigales, the prevailing ruminal anaerobic fungus, was almost undetectable after weaning using a RT-qPCR platform, confirming previous evidence that fungi colonize the rumen after the first week of life, while after weaning when ruminants fed with solid feed rich in starch, fungi almost disappeared (Fonty et al., 1987).

Although amylase activity was found to be higher at 45 d while protease increased at 100 d in P-fed lambs, there were no significant differences in animal performances. However, significant differences were found for the enzymatic activities from weaning to 100 d validating the maturation of rumen degradation potential over the time. It should be mentioned here, that collecting rumen digesta 4 hours after feeding was an optimal strategy to assess the amylolytic potential of lambs fed a grain-based diet, but also may be a possible explanation for the absence of alterations on fibrolytic enzymes. It has been reported that the degradation of plant cell wall

polysaccharides typically occurs in the later stages of ruminal fermentation, following the utilization of soluble carbohydrates and the subsequent buildup of the fibrolytic population (Martin and Michalet-Doreau, 1995).

The concentrations of VFAs were substantially increased in both dietary groups at 100 d compared to 45 d. Additionally, the molar proportion of acetate decreased, while the proportions of propionate and butyrate increased at 100 d compared to 45 d. These alterations align with the normal physiological changes in microbial activity observed during the growth of ruminants, reflecting the maturation of the rumen. Moreover, the concentration of VFAs were significantly higher at 100 d in the probiotic fed lambs indicating an improved microbial activity. In a previous meta-analysis, supplementation of *Saccharomyces cerevisiae* in growing goat diets resulted in higher concentration of total VFAs, especially propionate (Ogbuewu and Mbajiorgu, 2023), while in dairy cows the molar proportion of acetate was also increased (Cattaneo et al., 2023). The effect of *Saccharomyces cerevisiae* (CNCM I-1077) over ruminal VFA production has been also validated *in vitro* (Russouw et al., 2020). Further to a more stabilized rumen environment, which may lead to an increase in VFAs, it could be hypothesized that the rapidly fermentable carbohydrate diet resulted in lactate accumulation. In the case of P-fed lambs, this lactate could be utilized by *Selenomonas*, *Anaerovibrio*, and *Lachnospiraceae* (Louis and Flint, 2017) for propionate and butyrate formation, and by *Desulfovibrio* for acetate (Vita et al., 2015). This shift in ruminal biochemical functions regarding lactate utilization under the influence of probiotic yeasts has been extensively studied (Chaucheyras-Durand et al., 2008, Amin and Mao, 2021). An alternative integrative hypothesis proposes that the increased production of acetate and butyrate in probiotic-fed lambs may play a pivotal role in promoting uniform keratinization of the stratum corneum in the papillae. Additionally, these short-chain fatty acids could contribute to the advanced structural organization of papillae, particularly with respect to collagen and reticular fibers observed in probiotic-fed lambs. This effect could be linked to a reduction in osmotic pressure within the rumen epithelium, as suggested by Penner et al. (2009a). Besides, the promotive effects of butyrate on rumen epithelium development, particularly during early life, are well-documented (Amin et al., 2022). However, changes in VFA concentrations require further evaluation, as they are influenced by various factors, including VFA production, absorption, washout, and interconversion rate (Na and Guan, 2022), which were not assessed in this study. Interestingly, there is preliminary data supporting that cows experiencing a low severity ruminal acidosis had a greater gene expression of VFA metabolism regulators in rumen epithelia cells, which may result in higher VFA absorption rates, lowering the accumulation of VFA within the rumen (Penner et al., 2009b). Considering the molar proportion of VFAs in our study, the low acetate and high butyrate proportion in both dietary groups at the final sampling were also validating the influence of a diet rich in rapidly fermentable carbohydrates (Fonty and Chaucheyras-Durand, 2006).

Our observations on rumen histology, especially in mucosa, validated the observations in the rumen microbiota structure and altogether support the hypothesis of the protective role of the probiotic over the simulation of rumen dysbiosis. More specifically, our observations confirm that the P-fed lambs' mucosa histology outweighed that of the C-fed lambs as regards the appearance and distribution of the epithelium cell layers especially in the periphery of the papillae. In these regions, all layers presented distinctly and with same thickness, the extracellular matrix components (collagen and reticular fibers) that were more densely and uniformly packed in the mucosa, and the prekeratin and keratin that distinctly delineated epithelial pegs. The enhanced structure of the papillae in P-fed lambs may be attributed to the elevated concentration of butyrate in the rumen as explained above. Additional histochemical staining could further support these results, highlighting the positive effects of the treatment on rumen histology. In a previous study supplementing the same probiotic yeast with our work on finishing Charolais bulls, observations in the rumen wall unveiled that yeast might have acted as a preventive factor against hyperkeratinisation of the rumen papillae (Magrin et al., 2018). As reported in several studies on ruminants, the inclusion of different additives (probiotics such as yeasts, and prebiotics) in high-grain-based diets might enhance the health of the ruminal epithelium by reducing the stratum corneum thickness (Garcia Diaz et al., 2018).

The evidence presented supports the objective of this study, which aimed to induce dietary stress in lambs by incorporating highly fermentable starch through barley grains to simulate rumen dysbiosis, and subsequently evaluate the effects of probiotic live yeast. Indeed, when processing barley and corn similarly, the ruminal degradation rate and extent of starch and protein are faster and greater for barley compared to corn. This enhances the potential to cause digestive disorders, which could be detrimental to overall performance (DelCurto-Wyffels et al., 2021). Additionally, both wheat and barley have higher amylopectin content compared to corn, which can be fermented in the rumen at a greater rate (McCann et al., 2016; Shen et al., 2020). In several studies, supplementing probiotic yeast increased the ruminal pH and inhibited lactic acid accumulation either through the increased activity of lactate-utilizing microbes or via suppressed growth of lactate-producing species (Amin and Mao, 2021). Hence, it was plausible to expect significantly lower values of ruminal pH in C-fed lambs, especially at 100 d. Contradicting our hypothesis, pH was significantly altered only amongst the two sampling times. One hypothesis for the lack of LY's pH-balancing properties in the rumen could be attributed to the higher VFA concentrations observed in P-fed lambs at 100 d. The elevated VFA levels in these lambs may have contributed to a reduction in ruminal pH, potentially obscuring differences between the treatment groups. Although ruminal pH is considered to be the most trustable index of rumen digestive disorders, many biases could mislead the results with the most prevailing being the sampling method of rumen content. It has been reported several times within the literature that although stomach tubing is an alternative method to cannulation for collecting rumen digesta, it could be accompanied by a few biases (van Gastelen et al., 2019). Saliva contamination is a crucial factor that should be considered; indeed, in our study, the first quantity of rumen digesta was withdrawn aiming to minimize the contamination. Additionally, it has been also reported that pH is an important but not the only key factor driving the onset of SARA (Calsamiglia et al., 2008). A recent critical review by Golder and Lean (2024) highlights the limitations of relying on rumen pH as a sole diagnostic parameter for ruminal metabolic disorders, a practice now seen with increasing skepticism. It is now clear that pH variability within the rumen, combined with the challenges of probe accuracy and measurement drift in indwelling devices, significantly increases the potential for error in pH assessments. However, another study pointed out that despite showing a higher pH of the rumen content, the esophageal tubing did not introduce major differences in microbiota composition as compared to the whole content collection through the rumen cannula (da Cunha et al., 2023). Besides, although comparisons of pH

among different studies should be avoided when samples are not collected using the same sampling procedure due to the aforementioned reasons, the comparison of fingerprinting of microbiome structure is still valid (Terré et al., 2013). Interestingly, it seems that rumen fluid collection through esophageal tube may not have been the only obstacle to observe the expected changes in the pH. In a study where cows were subjected to a high starch diet following feeding challenge with 3 kg corn grain before morning feeding via rumen cannula, inactivated dry *S. cerevisiae* did not affect ruminal pH despite a decrease in lactate accumulation, even with samples collected directly through the cannula. This effect could be attributed to the fact that viability of the supplemented yeast may have an important impact on rumen stabilization.

5. Conclusions

The inclusion of live yeast *S. cerevisiae* CNCM I-1077 supported rumen microbiota stability and mitigated the extensive, nonuniform keratinization of the stratum corneum induced by a diet rich in fermentable carbohydrates. These changes were accompanied by increased concentrations of acetate, propionate, and butyrate, indicating enhanced energy metabolism in the probiotic-fed lambs. In the present study, the stomach tubing method for measuring ruminal pH was ineffective in predicting the observed changes in the ruminal ecosystem or accurately assessing the severity of the dysbiotic condition. Future studies should explore whether these histological changes influence lifelong performance and health.

CRediT authorship contribution statement

Kyriakaki Panagiota: Writing – review & editing, Methodology. **Cremonesi Paola:** Writing – review & editing, Methodology. **Petropoulos Konstantinos:** Writing – review & editing, Methodology. **Christodoulou Christos:** Writing – review & editing, Methodology. **Kotsampasi Basiliki:** Writing – review & editing, Methodology. **Dunière Lysiane:** Writing – review & editing, Methodology. **Balaskas Christos:** Writing – review & editing, Methodology. **Castiglioni Bianca:** Writing – review & editing, Methodology. **Tsiplakou Eleni:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Mavrommatis Alexandros:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Chevaux Eric:** Writing – review & editing, Funding acquisition, Conceptualization. **Severgnini Marco:** Writing – review & editing, Methodology.

Declaration of Competing Interest

The authors declare no conflict of interest. E. Chevaux and L. Dunière are employees of Lallemand SAS.

Acknowledgement

We would like to express our sincere gratitude to Dr. Frédérique Chaucheyras-Durand for her insightful feedback and thoughtful revisions of the manuscript.

Animal statement

Animal handling procedures were performed following protocols approved by the Agricultural University of Athens Ethical Committee of the Faculty of Animal Sciences (Number approval; 44/27–09–2021).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2025.116269.

References

Amin, A.B., Mao, S., 2021. Influence of yeast on rumen fermentation, growth performance and quality of products in ruminants: a review. Anim. Nutr. 7 (1), 31–41.
Amin, A.B., Trabi, E.B., Zhu, C., Mao, S., 2022. Role of butyrate as part of milk replacer and starter diet on intestinal development in pre-weaned calves. A systematic review. Anim. Feed Sci. Technol. 292, 115423.
Arshad, M.A., Hassan, F.U., Rehman, M.S., Huws, S.A., Cheng, Y., Din, A.U., 2021. Gut microbiome colonization and development in neonatal ruminants: strategies, prospects, and opportunities. Anim. Nutr. 7 (3), 883–895.
Bach, A., Iglesias, C., Devant, M., 2007. Daily rumen pH pattern of loose-housed dairy cattle as affected by feeding pattern and live yeast supplementation. Anim. Feed Sci. Technol. 136, 146–153.
Bahl, M.I., Bergström, A., Licht, T.R., 2012. Freezing fecal samples prior to DNA extraction affects the Firmicutes to Bacteroidetes ratio determined by downstream quantitative PCR analysis. FEMS Microbiol. Lett. 329, 193–197.
Belanche, A., Yáñez-Ruiz, D.R., Detheridge, A.P., Griffith, G.W., Kingston-Smith, A.H., Newbold, C.J., 2019. Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. Environ. Microbiol. 21, 4360–4377.
Belanche, A., Palma-Hidalgo, J.M., Nejjam, I., Jimenez, E., Martin-Garcia, A.I., Yanez-Ruiz, D.R., 2020. Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. J. Dairy Sci. 103 (6), 5047–5060.

Berg Miller, M.E., Antonopoulos, D.A., Rincon, M.T., Band, M., Bari, A., Akraiko, T., Hernandez, A., Thimmapuram, J., Henrissat, B., Coutinho, P.M., Borovok, I., Jindou, S., Lamed, R., Flint, H.J., Bayer, E.A., White, B.A., 2009. Diversity and strain specificity of plant cell wall degrading enzymes revealed by the draft genome of *Ruminococcus flavefaciens* FD-1. PLoS One 4, e6650.
Bi, Y., Zeng, S., Zhang, R., Diao, Q., Tu, Y., 2018. Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. BMC Microbiol. 18, 69.
Brede, M., Orton, T., Pinior, B., Roch, F.F., Dzieciol, M., Zwirzitz, B., Wagner, M., Breves, G., Wetzels, S.U., 2020. PacBio and Illumina MiSeq amplicon sequencing confirm full recovery of the bacterial community after subacute ruminal acidosis challenge in the RUSITEC system. Front. Microbiol. 11, 1813.
Brooke, C.G., Najafi, N., Dykier, K.C., Hess, M., 2019. *Prevotella copri*, a potential indicator for high feed efficiency in western steers. Anim. Sci. J. 90, 696–701.
Cabral, L.D.S., Weimer, P.J., 2024. *Megasphaera elsdenii*: Its role in ruminant nutrition and its potential industrial application for organic acid biosynthesis. Microorganisms 12 (2024), 219.
Calsamiglia, S., Cardozo, P.W., Ferret, A., Bach, A., 2008. Changes in rumen microbial fermentation are due to a combined effect of type of diet and pH. J. Anim. Sci. 86 (3), 702–711.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7 (5), 335–336.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. USA 108 (1), 4516–4522, 15.
Carberry, C.A., Kenny, D.A., Han, S., McCabe, M.S., Waters, S.M., 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Appl. Environ. Microbiol. 78, 4949–4958.
Cattaneo, L., Lopreiato, V., Piccioli-Cappelli, F., Trevisi, E., Minuti, A., 2023. Effect of supplementing live *Saccharomyces cerevisiae* yeast on performance, rumen function, and metabolism during the transition period in Holstein dairy cows. J. Dairy Sci. 106 (6), 4353–4365.
Chang, H.W., Nam, Y.D., Sung, Y., Kim, K.H., Roh, S.W., Yoon, J.H., An, K.G., Bae, J.W., 2007. Quantitative real time PCR assays for the enumeration of Saccharomyces cerevisiae and the Saccharomyces sensu stricto complex in human feces, 2007 Dec J. Microbiol Methods 71 (3), 191–201. https://doi.org/10.1016/j.mimet.2007.08.013.
Chaucheyras, F., Fonty, G., Bertin, G., Salmon, J.M., Gouet, P., 1996. Effects of a strain of Saccharomyces cerevisiae (Levucell SC), a microbial additive for ruminants, on lactate metabolism in vitro. Can. J. Microbiol. 42, 927–933.
Chaucheyras-Durand, F., Fonty, G., 2001. Establishment of cellulolytic bacteria and development of fermentative activities in the rumen of gnotobiotically-reared lambs receiving the microbial additive Saccharomyces cerevisiae CNCM I-1077. Reprod. Nutr. Dev. 41, 57–68.
Chaucheyras-Durand, F., Fonty, G., 2002. Influence of a Probiotic Yeast (Saccharomyces cerevisiae CNCM I-1077) on Microbial Colonization and Fermentations in the Rumen of Newborn Lambs. Microb. Ecol. Health Dis. 14, 30–36.
Chaucheyras-Durand, F., Walker, N.D., Bach, A., 2008. Effects of active dry yeasts on the rumen microbial ecosystem: past, present and future. Anim. Feed Sci. Technol. 145, 5–26.
Chaucheyras-Durand, F., Ameilbonne, A., Auffret, P., Bernard, M., Mialon, M.M., Dunière, L., Forano, E., 2019. Supplementation of live yeast based feed additive in early life promotes rumen microbial colonization and fibrolytic potential in lambs. Sci. Rep. 9 (1), 19216.
Chen, X.L., Wang, J.K., Wu, Y.M., Liu, J.X., 2008. Effects of chemical treatments of rice straw on rumen fermentation characteristics, fibrolytic enzyme activities and populations of liquid- and solid-associated ruminal microbes in vitro. Anim. Feed Sci. Tech. 141, 1–14.
Commun, L., Mialon, M.M., Martin, C., Baumont, R., Veissier, I., 2009. Risk of subacute ruminal acidosis in sheep with separate access to forage and concentrate. J. Anim. Sci. 87, 3372–3379.
da Cunha, L.L., Monteiro, H.F., Figueiredo, C.C., Canisso, I.F., Bicalho, R.C., Cardoso, F.C., Weimer, B.C., Lima, F.S., 2023. Characterization of rumen microbiome and metabolome from oro-esophageal tubing and rumen cannula in Holstein dairy cows. Sci. Rep. 13, 5854.
DelCurto-Wyffels, H.M., Dafoe, J.M., Parsons, C.T., Boss, D.L., DelCurto, T., Wyffels, S.A., Van Emon, M.L., Bowman, J.G.P., 2021. Corn versus Barley in Finishing Diets: Effect on Steer Performance and Feeding Behavior. Animals 11 (4), 935.
Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J.E., Gonzalez-Recio, O., 2019. Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. Sci. Rep. 9, 11.
Denman, S.E., McSweeney, C.S., 2006. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. FEMS Microbiol. Ecol. 58, 572–582.
Edgar, R.C., 2016. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. bioRxiv. https://doi.org/10.1101/081257.
Ellison, M.J., Conant, G.C., Lamberson, W.R., Cockrum, R.R., Austin, K.J., Rule, D.C., Cammack, K.M., 2017. Diet and feed efficiency status affect rumen microbial profiles of sheep. Small Rumin. Res. 156, 12–19.
Elolimy, A.A., Arroyo, J.M., Batistel, F., Iakiviak, M.A., Loor, J.J., 2018. Association of residual feed intake with abundance of ruminal bacteria and biopolymer hydrolyzing enzyme activities during the peripartal period and early lactation in Holstein dairy cows. J. Anim. Sci. Biotechnol. 9, 1–10.
Fernando, S.C., Purvis, H., Najar, F., Sukharnikov, L., Krehbiel, C., Nagaraja, T., Roe, B.A., Desilvaet, U., 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. Appl. Environ. Microbiol. 76, 7482–7490.
Fonty, G., Chaucheyras-Durand, F., 2006. Effects and modes of action of live yeasts in the rumen. Biologia 61, 741–750.
Fuentes, M.C., Calsamiglia, S., Cardozo, P.W., Vlaeminck, B., 2009. Effect of pH and level of concentrate in the diet on the production of biohydrogenation intermediates in a dual-flow continuous culture. J. Dairy Sci. 92 (9), 4456–4466.
Garcia Diaz, T., Ferriani Branco, A., Jacovaci, F.A., Cabreira Jobim, C., Bolson, D.C., Pratti Daniel, J.L., 2018. Inclusion of live yeast and mannan-oligosaccharides in high grain-based diets for sheep: Ruminal parameters, inflammatory response and rumen morphology. PLoS One 13 (2), e0193313.
van Gastelen, S., Schumacher, F., Cone, J.W., Dijkstra, J., Pellikaan, W.F., 2019. In dairy cattle, the stomach tube method is not a feasible alternative to the rumen cannulation method to examine in vitro gas and methane production. Anim. Feed Sci. Technol. 256, 114259.
Golder, H.M., Lean, I.J., 2024. Ruminal acidosis and its definition: A critical review. J. Dairy Sci. 30 (24), 01095–01096. S0022-0302.
Khafipour, E., Li, S., Plaizier, J.C., Krause, D.O., 2009. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. Appl. Environ. Microbiol. 75, 7115–7124.
Kim, M., Park, T., Yu, Z., 2017. Invited Review—Metagenomic investigation of gastrointestinal microbiome in cattle. Asian-Australas. J. Anim. Sci. 30, 1515–1528.
Louis, P., Flint, H.J., 2017. Formation of propionate and butyrate by the human colonic microbiota. Enviro. Microbiol. 19 (1), 29–41.
Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5, 169–172.
Magrin, L., Gottardo, F., Fiore, E., Gianesella, M., Martin, B., Chevaux, E., Cozzi, G., 2018. Use of a live yeast strain of Saccharomyces cerevisiae in a high-concentrate diet fed to finishing Charolais bulls: effects on growth, slaughter performance, behavior, and rumen environment. Anim. Feed Sci. Technol. 241, 84–93.
Mao, S.Y., Zhang, R.Y., Wang, D.S., Zhu, W.Y., 2013. Impact of subacute ruminal acidosis (SARA) adaptation on rumen microbiota in dairy cattle using pyrosequencing. Anaerobe 24, 12–19.
Martin, C., Michalet-Doreau, B., 1995. Variations in mass and enzyme activity of rumen microorganisms: Effect of barley and buffer supplements. J. Sci. Food Agric. 67, 407–413.
Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G., Neufeld, J.D., 2012. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinforma. 13, 31.
Mavrommatis, A., Christodoulou, C., Kyriakaki, P., Skourtis, A., Kotsampasi, B., Christodoulou, V., Symeon, S., Karatzia, M., Karatosidi, D., Savvidou, S., Righi, F., Arsenos, G., Chevaux, E., Tsiplakou, E., 2024. The impact of probiotic live yeast in a barley grain-based diet on feed efficiency, carcass traits, and immune-oxidative status of artificially reared lambs. Anim. Feed Sci. Technol. 309, 115915.
Mavrommatis, A., Skliros, D., Flemetakis, E., Tsiplakou, E., 2021b. Changes in the rumen bacteriome structure and enzymatic activities of goats in response to dietary supplementation with *Schizochytrium* spp. Microorganisms 9, 1528.

Mavrommatis, A., Skliros, D., Sotirakoglou, K., Flemetakis, E., Tsiplakou, E., 2021a. The effect of forage-to-concentrate ratio on *Schizochytrium* spp.-supplemented goats: Modifying rumen microbiota. Animals 20 11 (9), 2746.
McCann, J.C., Luan, S., Cardoso, F.C., Derakhshani, H., Khafipour, E., Loor, J.J., 2016. Induction of subacute ruminal acidosis affects the ruminal microbiome and epithelium. Front. Microbiol. 7, 701.
Mickdam, E., Khiaosa-Ard, R., Metzler-Zebeli, B.U., Klevenhusen, F., Chizzola, R., Zebeli, Q., 2016. Rumen microbial abundance and fermentation profile during severe subacute ruminal acidosis and its modulation by plant derived alkaloids in vitro. Anaerobe 39, 4–13.
Muizelaar, W., Bani, P., Kuhla, B., Tapio, I., Yáñez-Ruiz, D., van Gastelen, S., 2020. Rumen fluid sampling via oral stomach tubing method. In: S. Danesh Mesgaran, H., Ferguson, L., Munksgaard, M., Kennedy, Terré, E., Humphries, D., Dijkstra, J., Kuhla, B., Dewhurst, R., Baumont, R. (Eds.), Methods in cattle physiology and behaviour research: Recommendations from the Smartcow consortium. https://doi.org/10.5680/mcpb008.
Na, S.W., Guan, L.L., 2022. Understanding the role of rumen epithelial host-microbe interactions in cattle feed efficiency. Anim. Nutr. 16 (10), 41–53.
Nisbet, D.J., Martin, S.A., 1991. Effect of a Saccharomyces cerevisiae culture on lactate utilization by the ruminal bacterium Selenomonas ruminantium. J. Anim. Sci. 69 (11), 4628–4633.
Oetzel, G.R., 2003. Subacute ruminal acidosis in dairy cattle. Adv. Dairy Technol. 15, 307–317.
Ogbuewu, I.P., Mbajiorgu, C.A., 2023. Meta-analysis of *Saccharomyces cerevisiae* on enhancement of growth performance, rumen fermentation and haemato-biochemical characteristics of growing goats. Heliyon 9 (3), e14178.
Pedersen, C., Ijaz, U.Z., Gallagher, E., Horton, F., Ellis, R.J., Jaiyeola, E., Duparc, T., Russell-Jones, D., Hinton, P., Cani, P.D., La Ragione, R.M., Robertson, M.D., 2018. Fecal Enterobacteriales enrichment is associated with increased in vivo intestinal permeability in humans. Physiol. Rep. 6 (7), e13649.
Penner, G.B., Aschenbach, J.R., Gäbel, G., Rackwitz, R., Oba, M., 2009a. Epithelial capacity for apical uptake of short chain fatty acids is a key determinant for intraruminal pH and the susceptibility to sub-acute ruminal acidosis in sheep. J. Nutr. 139, 1714–1720.
Penner, G.B., Taniguchi, M., Guan, L.L., Beauchemin, K.A., Oba, M., 2009b. Effect of dietary forage to concentrate ratio on volatile fatty acid absorption and the expression of genes related to volatile fatty acid absorption and metabolism in ruminal tissue. J. Dairy Sci. 92 (6), 2767–2781.
Petri, R.M., Schwaiger, T., Penner, G.B., Beauchemin, K.A., Forster, R.J., McKinnon, J.J., McAllister, T.A., 2013. Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. PLoS One 8 (12), e83424, 31.
Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41 (Database), D590–D596.
Ravelo, A.D., Arce-Cordero, J.A., Lobo, R.R., Liu, T., Jeong, K.C., Faciola, A., 2023. Effects of partially replacing dietary corn with sugars in a dual-flow continuous culture system on the ruminal microbiome. Transl. Anim. Sci. 7 (1), txad011.
Russell, J.B., Dombrowski, D.B., 1980. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. Appl. Environ. Microbiol.. 39, 604–610.
Russouw, A., Chevaux, E., Chaucheyras-Durand, F., Esposito, G., Raffrenato, E., 2020. Effects of Saccharomyces cerevisiae, medium and forage type and their interactions on in vitro ruminal fermentation. Heliyon 6 (9), e05028.
Shen, J., Zheng, L., Chen, X., Han, X., Cao, Y., Yao, J., 2020. Metagenomic analyses of microbial and carbohydrate-active enzymes in the rumen of dairy goats fed different rumen degradable starch. Front. Microbiol. 11, 1003.
Steele, M.A., AlZahal, O., Hook, S.E., Croom, J., McBride, B.W., 2009. Ruminal acidosis and the rapid onset of ruminal parakeratosis in a mature dairy cow: a case report. Acta Vet. Scand. 51, 39.
Suen, G., Stevenson, D.M., Bruce, D.C., Chertkov, O., Copeland, A., Cheng, J.F., Detter, C., 2011. Complete genome of the cellulolytic ruminal bacterium *Ruminococcus albus* 7. Am. Soc. Microbiol. 193, 5574–5575.
Sylvester, J.T., Karnati, S.K., Yu, Z., Morrison, M., Firkins, J.L., 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. J. Nutr. 134, 3378–3384.
Terré, M., Castells, L., Fàbregas, F., Bach, A., 2013. Short communication: Comparison of pH, volatile fatty acids, and microbiome of rumen samples from preweaned calves obtained via cannula or stomach tube. J. Dairy Sci. 96 (8), 5290–5294.
Vita, N., Valette, O., Brasseur, G., Lignon, S., Denis, Y., Ansaldi, M., Dolla, A., Pieulle, L., 2015. The primary pathway for lactate oxidation in *Desulfovibrio vulgaris*. Front. Microbiol. 6, 606.
Wang, M.Y., Li, Y., Gao, M., Song, L.W., Xu, M., Zhao, X.L., Jia, Y., Zhao, M., Sun, Y.Y., Hu, H.L., 2021. Effects of subacute ruminal acidosis on colon epithelial morphological structure, permeability, and expression of key tight junction proteins in dairy goats. J. Dairy Sci. 104 (4), 4260–4270.
Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol 73 (16), 5261–5267.
Whittaker, R.H., 1960. Vegetation of the Siskiyou Mountains, Oregon and California. Ecol. Monogr. 30 (3), 1960-07.
Yang, S.L., Bu, D.P., Wang, J.Q., Hu, Z.Y., Li, D., Wei, H.Y., Loor, J.J., 2009. Soybean oil and linseed oil supplementation affect profiles of ruminal microorganisms in dairy cows. Animal 3, 1562–1569.
Zhang, R., Ye, H., Liu, J., Mao, S., 2017. High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. Appl. Microbiol. Biotechnol. 101, 6981–6992.
Zhang, Z., Li, F., Ma, X., Li, F., Wang, Z., 2022. Effects of barley starch level in diet on fermentation and microflora in rumen of Hu sheep. Animals 12, 1941.
Zhao, C., Liu, G., Li, X., Guan, Y., Wang, Y., Yuan, X., Sun, G., Wang, Z., Li, X., 2018. Inflammatory mechanism of Rumenitis in dairy cows with subacute ruminal acidosis. BMC Vet. Res. 14, 135.