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Multi-omics *in vitro* study of the salivary modulation of the goat rumen microbiome

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ABSTRACT

Ruminants are able to produce large quantities of saliva which enter into the rumen and salivary components exert different physiological functions. Although previous research has indicated that salivary immunoglobulins can partially modulate the rumen microbial activity, the role of the salivary components other than ions on the rumen microbial ecosystem has not been thoroughly investigated in ruminants. To investigate this modulatory activity, a total of 16 semi-continuous *in vitro* cultures with oats hay and concentrate were used to incubate rumen fluid from four donor goats with autoclaved saliva (**AUT**) as negative control, saliva from the same rumen fluid donor (**OWN**) as positive control, and either goat (**GOAT**) or sheep (**SHEEP**) saliva as experimental interventions. Fermentation was monitored throughout 7 days of incubation and the microbiome and metabolome were analysed at the end of this incubation by Next-Generation sequencing and liquid chromatography coupled with mass spectrometry, respectively. Characterisation of the proteome and metabolome of the different salivas used for the incubation showed a high inter-animal variability in terms of metabolites and proteins, including immunoglobulins. Incubation with AUT saliva promoted lower fermentative activity in terms of gas production (−9.4%) and highly divergent prokaryotic community in comparison with other treatments (OWN, GOAT and SHEEP) suggesting a modulatory effect derived from the presence of bioactive salivary components. Microbial alpha-diversity at amplicon sequence variant (**ASV**) level was unaffected by treatment. However, some differences were found in the microbial communities across treatments, which were mostly caused by a greater abundance of *Proteobacteria* and *Rikenellacea* in the AUT treatment and lower of *Prevotellaceae*. These bacteria, which are key in the rumen metabolism, had greater abundances in GOAT and SHEEP treatments. Incubation with GOAT saliva led to a lower protozoal concentration and propionate molar proportion indicating a capacity to modulate the rumen microbial ecosystem. The metabolomics analysis showed that the AUT samples were clustered apart from the rest indicating different metabolic pathways were promoted in this treatment. These results suggest that specific salivary components contribute to host-associated role in selecting the rumen commensal microbiota and its activity. These findings could open the possibility of developing new strategies to modulate the saliva composition as a way to manipulate the rumen function and activity.

Implications

It is unclear whether the bioactive components of saliva vary across different animals and if the rumen microbiota is shaped differently because of this. Saliva from different goats, sheep and autoclaved saliva were incubated with goat rumen fluid. The use of saliva lacking bioactive components caused major changes in the fermentation pattern, microbial community and metabolomics profile. This, together with some differences found between incubating with goat or sheep saliva, speaks for the crucial role that distinct salivary components play in the regulation of the rumen microbiota and how that can also contribute to inter-species differences. These findings are a step forward into assessing different

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mechanisms by which the host can modulate the gut microbiota to optimise its digestive process.

Introduction

The rumen of ruminant animals contains a great diversity of prokaryotic (bacteria, archaea, virus) and eukaryotic (protozoa and fungi) microorganisms that together breakdown and ferment the feed ingested by the host animal to convert complex plant carbohydrates into short-chain volatile fatty acids (Dehority, 2003). The rumen microbial diversity and function largely influence many animal traits such as the efficiency of utilisation of feeds and the environmental impact through methane emissions (Jami et al., 2014). The digestive microbiome in most mammals are controlled by host genetic variation (Koskella and Bergelson, 2020; Li et al., 2019) through multiple processes, one of the most crucial being immune modulation, by the presence of bioactive metabolites secreted from epithelial cells (i.e. antimicrobial peptides, immunoglobulins, etc.) and germline-encoded pattern recognition receptors (Zheng et al., 2020). However, the rumen epithelium includes up to a 15 cell layer that limit the permeability of large molecules, and lacks an organised lymphoid tissues (Sharpe et al., 1977). To circumvent this limitation, saliva seems to be the main vehicle of introducing immune active metabolites in to the rumen (Yáñez-Ruiz et al., 2015).

Ruminants' saliva is secreted in large amounts and assists the animal in the process of feed lubrication, deglutition and regurgitation (Mcdougall, 1948; Somers, 1957). Saliva constituents include a significant amount of ions (mainly bicarbonate and phosphate), that help maintain rumen osmotic pressure and pH within the physiological range (Warner and Stacy, 1977) providing a buffered medium to allow rumen microorganisms to thrive (Faniyi et al., 2019). The protein fraction of saliva comprises a number of proteins involved in transportation and pH buffering (Cheaib and Lussi, 2013), from which albumin is found in greater amounts (Lamy et al., 2009). However, the ruminant salivary proteome also includes a complex mix of other proteins with a wide range of physiological and enzymatic functions (Ang et al., 2011). Immunoglobulins, especially secretory immunoglobulin A (**IgA**), modulates the proliferation of symbiotic microbiota (Fouhse et al., 2017), either inhibiting or stimulating their growth (Donaldson et al., 2018). Smaller salivary proteins, which includes a variety of cytokines (Stenken and Poschenrieder, 2015) and antimicrobial peptides (Fábián et al., 2012), have been shown to be the most discriminant in the salivary proteome across individuals and animal species (Lamy et al., 2009). In a recent *in vitro* batch culture study, we have shown that some specific protein components have the ability to modulate rumen fermentation in goats (Palma-Hidalgo et al., 2021a). However, due to such specificity, the mechanisms behind the complex and modulatory interaction that takes place between salivary components and host rumen microbiota are still largely unknown.

This work aimed to assess the role of saliva on modulating the rumen fermentation and microbial profiles using a 7-day semi-continuous *in vitro* trial. A detailed characterisation of the protein and metabolites composition of saliva from different individual animals was conducted and rumen fluid from goats was incubated with different types of saliva (own animal saliva (**OWN**), different goat saliva (**GOAT**), sheep saliva (**SHEEP**) and autoclaved saliva (**AUT**)) to elucidate the potential modulatory effect on the rumen microbial ecosystem.

Our hypothesis was that the bioactive components of saliva would foster the rumen fermentative activity and that the incubation with saliva from different individuals or animal species would result in changes in the *in vitro* rumen microbial, fermentative and metabolomics profiles.

Material and methods

Saliva collection

Experimental protocols were approved by the Ethical Committee for Animal Research at EEZ-CSIC, and animal procedures were conducted by trained personnel according to the Spanish Animal Experimentation guidelines (RD 53/2013). Five healthy female rumen-cannulated goats (Goat 1 to Goat 5) and one female sheep with similar age (~4 years), all housed in different pens, were used as saliva donors. During the study, all animals were fed at the maintenance level with a diet consisting of 80% oats hay and 20% commercial concentrate. Saliva collection was conducted before the morning feeding by swabbing the base of the cheek on both sides of the mouth of the animals with absorbent sponges for 5 min. Saliva was collected from the sponges by centrifuging at 190*g* for 10 min, then filtrated through 0.25 μm pore size to remove microorganisms and large particles, pooled per animal and stored in aliquots at −80 °C until the start of the *in vitro* incubation and subsequent days. Additionally, equal volumes of saliva from goats 1–4 were mixed, autoclaved at 121 °C for 30 min and stored at −80 °C (AUT). Four aliquots from each saliva (goats 1–5 and sheep) were used for immunoglobulins, proteome and metabolome analyses.

Experimental design and in vitro incubation

A 7-day *in vitro* semi-continuous incubation was conducted using rumen fluid from four goats (Goats 1–4) sampled before the morning feeding and filtrated through a double layer of cheesecloth. Sixteen Wheaton bottles with 30 ml capacity were used in the incubation. Each rumen fluid (n = 4) was placed in four bottles and incubated with different types of saliva: OWN treatment consisted of saliva from the same rumen fluid donor animal (Goats 1–4) as positive control, GOAT treatment consisted of saliva from goat 5, SHEEP treatment consisted on saliva from sheep and AUT treatment consisted on pooled autoclaved saliva (from Goats 1 to 4) to denature active metabolites in the saliva but keeping the minerals, as negative control (Fig. 1). Incubations consisted of 20 ml volume per bottle composed by 6.67 mL of rumen fluid, 6.67 mL of saliva and 6.67 mL of bicarbonate buffer (3.5 g $NaHCO_3$ + 0.4 g $(NH_4)HCO_3$ in 100 ml dH_2O). The same oats hay and commercial concentrate that were offered to the animals were ground to 1 mm size particles and used as incubation substrate (100 mg each) to simulate a typical diet in small ruminants intensive systems.

In order to maintain an active *in vitro* system, every 12 hours (0900 h and 2100 h), the gas pressure in the headspace of the bottles was measured using a Wide Range Pressure Meter (SperScientific Ltd, Scottsdale, AZ, USA), which then was transformed into volume units by the ideal gas law. After gas measurement, bottles were opened, the content was homogenised by a gentle horizontal movement and 1/3 of the incubation volume (6.67 ml) was removed with a syringe and used to measure the pH. The removed incubation volume was replaced by 3.33 ml of the aforementioned bicarbonate buffer, 3.33 ml of the same saliva used in each treatment (stored at −80 °C and thawed at room temperature), and 1/3 of diet (33 mg oats hay and 33 mg commercial concentrate). A continuous flow of CO_2 was applied to each bottle through this process to maintain the anaerobic conditions. One sub-sample (800 μl) of the removed incubation content was taken at 12, 36, 60, 84, 108, 132 and 156 hours (days 1, 2, 3, 4, 5, 6 and 7; respectively), mixed with 800 μL of an acid solution (0.5 mol/l HCl, 200 g/l metaphosphoric acid and 0.8 g/l crotonic acid as internal standard) for volatile fatty acid (**VFA**) determination by gas chromatography (AutoSystem gas chromatograph, Perkin Elmer, Waltham,

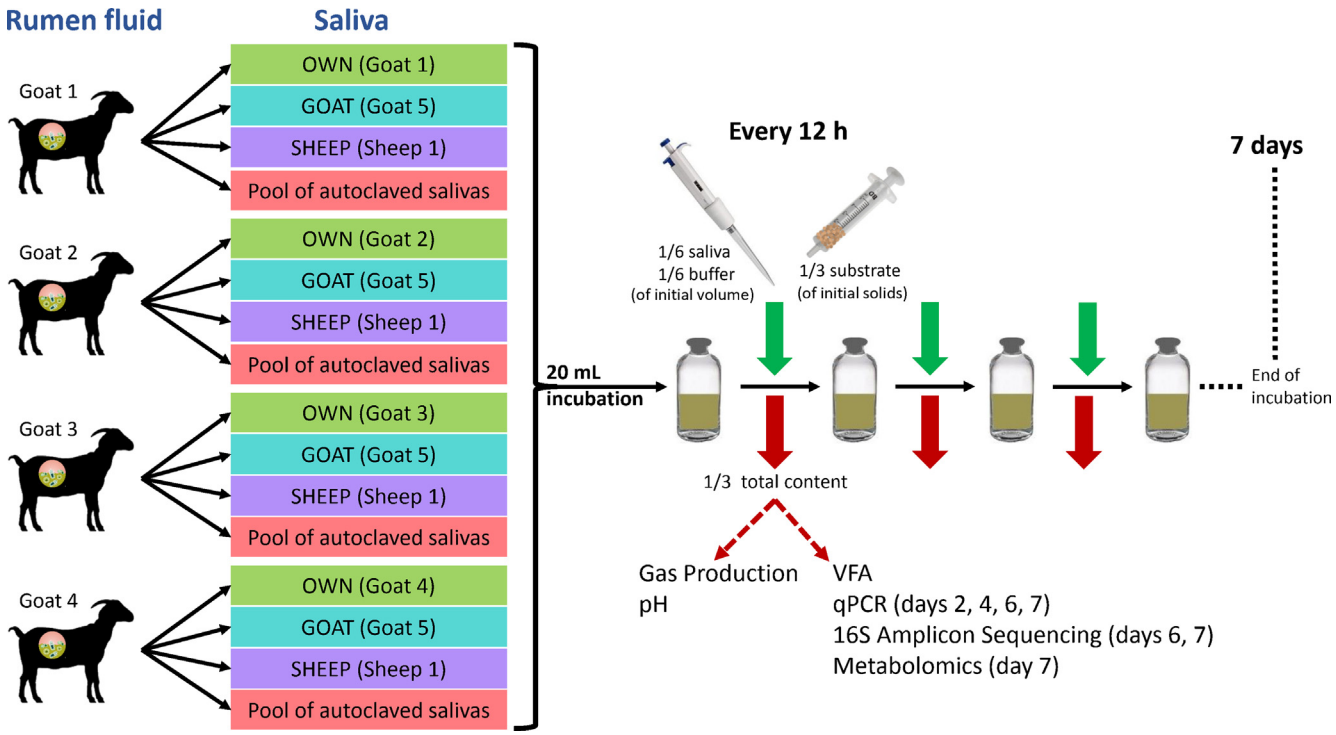

Fig. 1. Experimental design of the study. Rumen fluid from four goats was incubated with either their own saliva, saliva from a different goat, saliva from sheep or a pool of autoclaved saliva for 7 days in a 20 ml incubation volume. Every 12 hours, 1/3 of the incubation content was removed and replaced by equal volumes of buffer (1/6 of initial volume) and each kind of saliva (1/6 of initial volume) and 1/3 of the initial substrate. VFAs = Volatile Fatty Acids. qPCR = quantitative PCR.

MA). A second sub-sample (200 µl) of the removed incubation content at day 7 was used for metabolomics analysis following a similar procedure to that described for saliva samples. A third sub-sample (200 µl) taken on days 2, 4, 6 and 7 was used for DNA extraction using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Ltd., Barcelona, Spain).

Characterisation of proteins and metabolites in saliva

One aliquot of each saliva was thawed to measure the protein content by spectrophotometry using a commercial assay kit (PierceTM BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). For salivary immunoglobulins A and G (**IgG**) quantification, aliquots of each saliva were thawed and centrifuged at 3 000*g* for 10 min. IgA and IgG concentrations were measured using the Goat Immunoglobulin A and Goat Immunoglobulin G ELISA kits (MyBioSource, San Diego, CA, USA), respectively.

Before conducting saliva proteome analysis, albumin depletion was performed on thawed aliquots of each saliva, using the Pierce™ Albumin Depletion Kit (Thermo Fisher Scientific, Waltham, MA, USA) in order to reduce the high concentrations of albumin in saliva. After that, albumin-depleted saliva samples were sent to Proteomics Service at Instituto de Parasitología y Biomedicina López Neyra (IPBLN-CSIC, Granada, Spain) for non-targeted proteomic analysis using a nano liquid chromatograph (Easy nano Liquid Chromatograph, Proxeon, Odensee, Denmark) coupled with an Amazon Speed ETD ion trap mass spectrometer fitted with CaptiveSpray ion source (Bruker, Bremen, Germany). Saliva samples were processed and analysed as described by Mancera-Arteu et al., (2020). Identified spectra were searched against the TrEMBL database (Bateman et al., 2021). Exponentially modified protein abundance index (**emPAI**), which is proportional to protein content in a protein mixture (Ishihama et al., 2005), was used for estimation of absolute protein amount in the saliva samples (Arike and Peil, 2014).

Saliva aliquots for non-targeted metabolomics analysis were sent to the Metabolomic Platform at Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, Madrid, Spain). Metabolites were extracted and injected in an Ultrahigh-pressure liquid chromatograph (Agilent 1290 Infinity UHPLC, Santa Clara, CA, USA) coupled with a Quadrupole Time-Of-Flight Mass Spectrometer (Agilent 6540 UHD Q-TOF MS, Santa Clara, CA, USA) in a similar manner as described by Gómez et al. (2016). The tandem mass spectrometry spectra were processed and filtrated using MS-DIAL v 4.12 software (https://prime.psc.riken.jp/compms/msdial/main.html) and identified by searching against NIST (https://www.nist.gov/pml/atomic-spectra-database), MoNA (https://mona.fiehnlab.ucdavis.edu/) and LipidBlast (https://fiehnlab.ucdavis.edu/projects/lipidblast) databases. The sum peak height of all structurally annotated compounds (**mTIC**) score (Fiehn, 2017) was normalised for each sample to allow comparisons across saliva samples.

Next-generation sequencing and quantitative PCR

After extraction, DNA concentration and purity were assessed at A260 and A280nm on a NanoDrop™ OneC Microvolume UV–vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Eluted DNA (2 µl) were used to assess the abundance of the main microbial groups by quantitative PCR using an iQ5 multicolor Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA, USA). Specific primers for the 16S rRNA gene for bacteria (GTGSTGCAYGGYTGTCGTCA forward and CGTCRTCCMCACCTTCCTC reverse; Maeda et al., 2003), *mcrA* (Methyl-coenzyme M reductase) gene for methanogenic archaea (TTCGGTGGATCDCARAGRGC forward and GBARGTCGWAWCCGTAGAATCC reverse; Denman et al., 2007) and 18S rRNA genes for protozoa (GCTTTCGWTGGTAGTGTATT forward and CTTGCCCTCYAATC GTWCT reverse; Sylvester et al., 2004;) and

anaerobic fungi (GAGGAAGTAAAAGTCGTAACAAGGTTTC forward and CAAATTCACAAAGGGTAGGATGAT reverse; Denman and McSweeney, 2006; respectively) were used. Cycling conditions were 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 55 s; and 72 °C for 1 min. The absolute amount of each microbial group, expressed as DNA copies/ml of fresh matter, was determined using serial dilutions of known amounts of standards. Quantitative PCR standards consisted of the plasmid PCR 4-TOPO from *E. coli* (Invitrogen, Carlsbad, CA, USA), with an inserted 16S, *mcrA* or 18S rRNA gene fragment from each microbial group, respectively.

Extracted DNA from incubation samples taken on days 6 and 7 were also used for meta-taxonomic analysis of the prokaryotic community. DNA samples were sent to the Genomics Service at Instituto de Parasitología y Biomedicina López Neyra (IPBLN-CSIC, Granada, Spain) for amplicon sequencing using the library preparation protocol and Miseq V3 (600 cycles) kit provided by Illumina (Illumina Inc., San Diego, CA, USA). Primers used for the amplification were 5′-CCTACGGGNBGCASCAG-3′ and reverse: 5′-GAC TACNVGGGTATCTAATCC-3′ targeting the V3-V5 hypervariable region of the prokaryotic 16S rRNA gene (Takahashi et al., 2014). Paired-end reads were demultiplexed and had primer sequences removed using QIIME 2 (Bolyen et al., 2019). Reads were merged, denoised and chimera checked using the DADA2 plugin (Callahan et al., 2016). Amplicon sequence variants (**ASVs**) were identified, and then, taxonomy was assigned to ASVs using the q2-feature-classifier. (Bokulich et al. 2018) classify-sklearn naïve Bayes taxonomy classifier against the Silva 132 reference sequences at 99% identity (Quast et al., 2013). Once the alignment was performed, the number of sequences per sample for each microbial group was normalised across all the samples and singletons were removed. Raw sequence reads were deposited at the European Nucleotide Archive repository (accession: PRJEB45956) (Palma-Hidalgo et al., 2021b).

Calculations and statistical analysis

Statistical analyses were carried out using SPSS software (IBM Corp., version 26.0, New York, USA). To assess the effect of time on the fermentative activity throughout the incubation, rumen fermentation parameters were analysed based on a repeated measures mixed effects ANOVA as follows:

$$Y_{ijk} = \mu + S_i + T_j + ST_{ij} + R_k + e_{ijk}$$

where Y_{ijk} is the dependent, continuous variable, μ is the overall population of the mean, S_i is the fixed effect of the type of saliva (AUT vs OWN vs GOAT vs SHEEP), T_j is the fixed effect of the time (12 h vs 36 h vs 60 h vs 84 h vs 108 h vs 132 h vs 156 h sampling times), ST_{ij} is the interaction term, R_k is the random effect of the rumen fluid and e_{ijk} is the residual error. To assess only the effect of the type of saliva used in the incubation when this became stable, rumen fermentation parameters, quantitative PCR data and microbial taxa abundances at days 6 and 7 and incubation metabolites at day 7 were analysed using an ANOVA test with the saliva treatment (AUT vs OWN vs GOAT vs SHEEP) as fixed effect and the sampling times as a block. When significant effects were detected, means were compared by Fisher's protected LSD-test. Quantitative PCR data and microbial relative abundances were log10 transformed before the analysis to achieve a normal distribution. Only prokaryotic families and genera with relative abundance >0.1% across saliva treatments (in % of total sequences) were further considered for taxonomic analyses. In all analyses, significant effects were declared at $P < 0.05$ and the tendency to difference at $P < 0.1$.

Proteomics and metabolomics heatmaps based on emPAI and mTIC values, respectively, were constructed using the *stats* package in R (R Foundation for Statistical Computing, Vienna, Austria) to characterise the saliva samples before incubation and the effect of saliva on the rumen metabolome at 156 h of *in vitro* incubation. A Permutation-based Analysis of Variance (**PERMANOVA**) with 999 random permutations based on the Bray Curtis Dissimilarity Matrix was performed based on the mTIC values to compare the metabolomes across treatments using PAST software (Hammer et al., 2001). A Venn diagram was performed to illustrate the saliva treatment effects on the microbial community at ASV level using a multiple list comparator (https://www.molbiotools.com). To illustrate the treatment impact on the *in vitro* rumen prokaryotic community, a PERMANOVA based on the Bray Curtis Dissimilarity Matrix was performed on log10 transformed sequencing data with 999 random permutations using the vegan package within R. Pair-wise comparisons were performed to compare the microbial composition across treatments. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was carried out on center log-ratio transformed sequencing data to show the effect of the treatment on the prokaryotic communities' structure using mixOmics package in R. Furthermore, boxplots based on the relative abundance of the 10 most abundant bacterial families were constructed. Additional Spearman correlations (ρ) were calculated to assess the relationships between the microbial taxa abundance (log10 number of sequences) and the fermentation parameters, quantitative PCR data and metabolites mTIC values. Strong correlations were defined as those with $\rho \geq 0.4$ or ≤ -0.4.

Results

Immunological, proteomic and metabolomic profiling of the individual saliva samples

Saliva samples from the OWN (goats 1–4), GOAT (goat 5) and SHEEP treatments showed distinct immunological, proteomic and metabolomic profiles. Average protein concentration across saliva samples was 908 ± 146.2 μg/ml. IgA Elisa resulted in a salivary IgA concentration of 36.6, 30.0, 26.3 and 57.3 μg/ml in Goats 1–4, respectively, in comparison with 44.6 μg/ml (+19% compared to average OWN) in GOAT and 29.1 μg/ml (−22%) in the SHEEP treatment. In the case of salivary IgG quantification, the concentration in Goats 1–4 was 9.98, 9.72, 11.7 and 11.5 μg/ml, respectively; similar to that in GOAT (10.48 μg/ml), but much higher (+24%) than noted in SHEEP (8.12 μg/ml).

The proteomic tandem mass spectrometry analysis of the saliva samples resulted in the identification of 195 proteins/polypeptides across the six samples. The average number of proteins per sample was 59, with the GOAT saliva having the lowest count (46), and the SHEEP saliva having the highest (68). The heatmap of the 30 most abundant proteins across the saliva samples showed a very variable proteomic profile based on emPAI values (Fig. 2). On average, the protein with the greatest abundance was Thymosin beta, but its emPAI values were very variable, ranging from 0 (Goat 2) to 153 (Goat 3). Even after saliva samples were processed for albumin depletion, albumin (fragment) was still the second most abundant protein in the saliva samples. The 3rd (Beta A globin chain), 4th (II alpha globin) and 5th (I alpha globin) most abundant proteins were all haemoglobin subunits, and their abundance pattern was similar in each saliva sample. The variability in the abundance of the rest of the proteins helped determine, to some extent, the clustering pattern between the six samples. Interestingly, saliva from Goat 3 was identified as the most different across all samples with regard to the proteomic profile. However, unlike all the goats' salivas, SHEEP saliva did not contain goat-specific Capra hircus Akirin 2 mRNA but it did have high values of other proteins such as

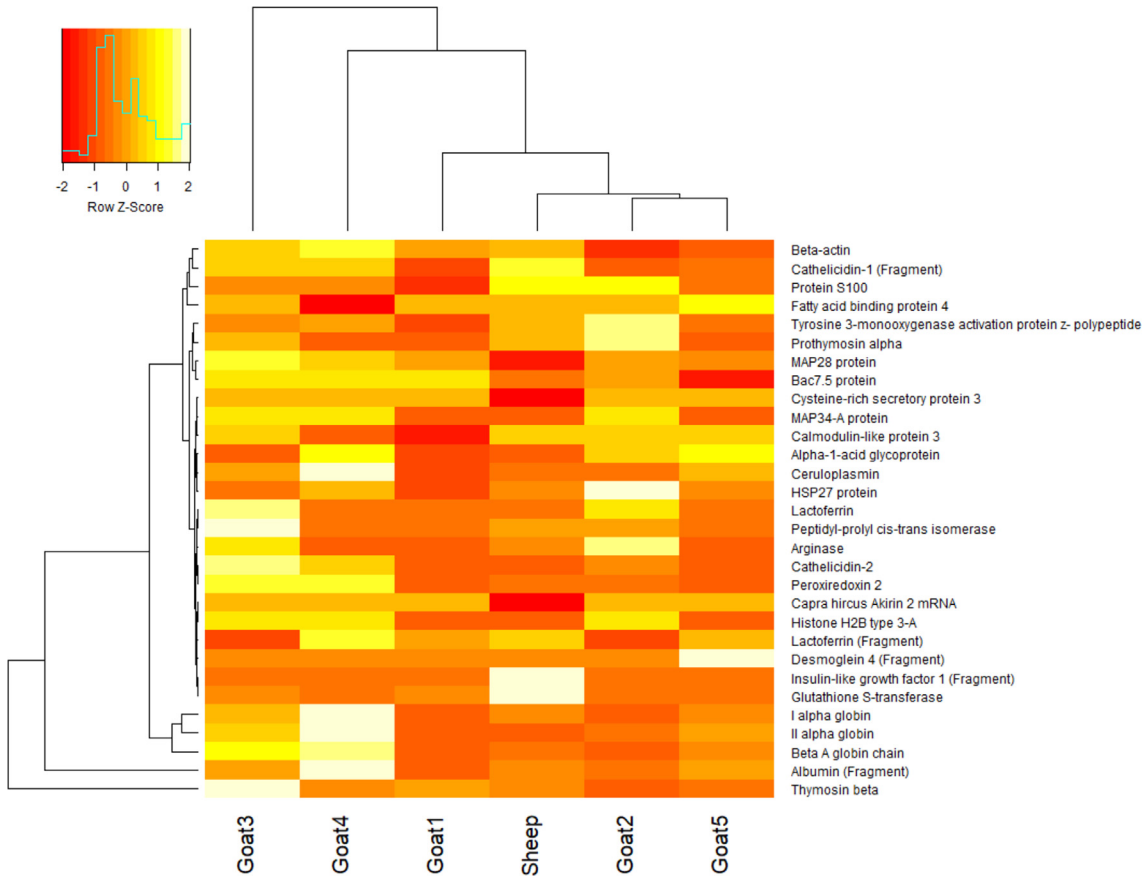

Fig. 2. Heatmap showing the abundance based on emPAI (exponentially modified protein abundance index) values of the 30 most abundant proteins/polypeptides found across the saliva samples (from Goats 1 to 5 and one sheep) used in the *in vitro* incubation.

Glutathione S-transferase and Insulin-like growth factor 1, which were almost not present in goat saliva samples.

The metabolomic tandem mass spectrometry analysis identified 39 metabolites present in all the saliva samples. The heatmap of the 39 compounds based on mTIC values showed very unique metabolic profiles in each saliva sample (Fig. 3). The peak heights of most of the compounds detected in the mass spectrometry spectra were very variable across the 6 samples, hence the high degree of variability observed in the constructed heatmap. Similar to what was found in the proteomic analysis, the Goat 3 had, again, the most divergent saliva sample in terms of metabolomic profile. The 1st (Tetraethylene glycol), 4th (Hexaethylene glycol) and 5th (Deithylene glycol monoethyl ether) most abundant metabolites on average were ethylene glycol derivatives. Other abundant detected compounds include the aminoacids L-Isoleucine (2nd) and L-Phenylalanine (8th), as well as the choline cation (3rd) and the Tri(3-chloropropyl) phosphate (6th), all of them with up to 1 000 fold mTIC value variability between at least two of the saliva samples. In comparison to GOAT saliva, the SHEEP saliva had lower concentrations of urea and higher concentrations of creatinine and nucleic acid derivatives such as guanine, guanosine or hypoxantine.

In vitro fermentation and microbial abundances

The fermentative activity peak was reached in the first 12 hours of incubation, as shown by the lowest pH values and greatest gas production and VFA concentration ($P < 0.001$ according to sampling time). From the first day of incubation, a stable fermentative activity was observed in terms of pH and gas production, with only a slight gradual decrease in total VFA concentration as incubation time progressed (Supplementary Table S1). The incubation of rumen fluid from four different goats with AUT, OWN, GOAT and SHEEP saliva led to substantial differences in the fermentation pattern (Table 1). Incubation with AUT had the lowest gas production (−9.4%) and highest pH and butyrate molar proportion (+6.4%), while GOAT samples produced the lowest pH, butyrate molar proportion and acetate: propionate ratio ($P < 0.001$, $P = 0.018$ and $P = 0.005$; respectively). Bottles with SHEEP saliva generated the highest gas production and highest acetate: propionate ratio (+4 and +6%; respectively) across treatments.

Results from quantitative PCR analyses showed a higher concentration of bacteria in bottles incubated with SHEEP and OWN saliva, in comparison to those from AUT and GOAT treatments ($P = 0.013$, Table 1). Likewise, the highest protozoal concentration was found in SHEEP and the lowest in GOAT bottles ($P = 0.046$). No significant effects were noted on the methanogenic archaea or anaerobic fungi concentrations across treatments.

Microbial diversity and community structure

The sequencing analysis performed on incubation samples generated 41 514 ± 13 383 high-quality prokaryotic sequences per sample. The number of sequences was normalised to 28 131 for further processing and analyses. Good's coverage index was 98.8 ± 0.006% on average and similar for the four saliva treatments, hence, a good level of sequencing depth was achieved. The primers used for sequencing mostly targeted bacterial amplicons, however, ∼0.75% of the detected reads were identified as archaeal

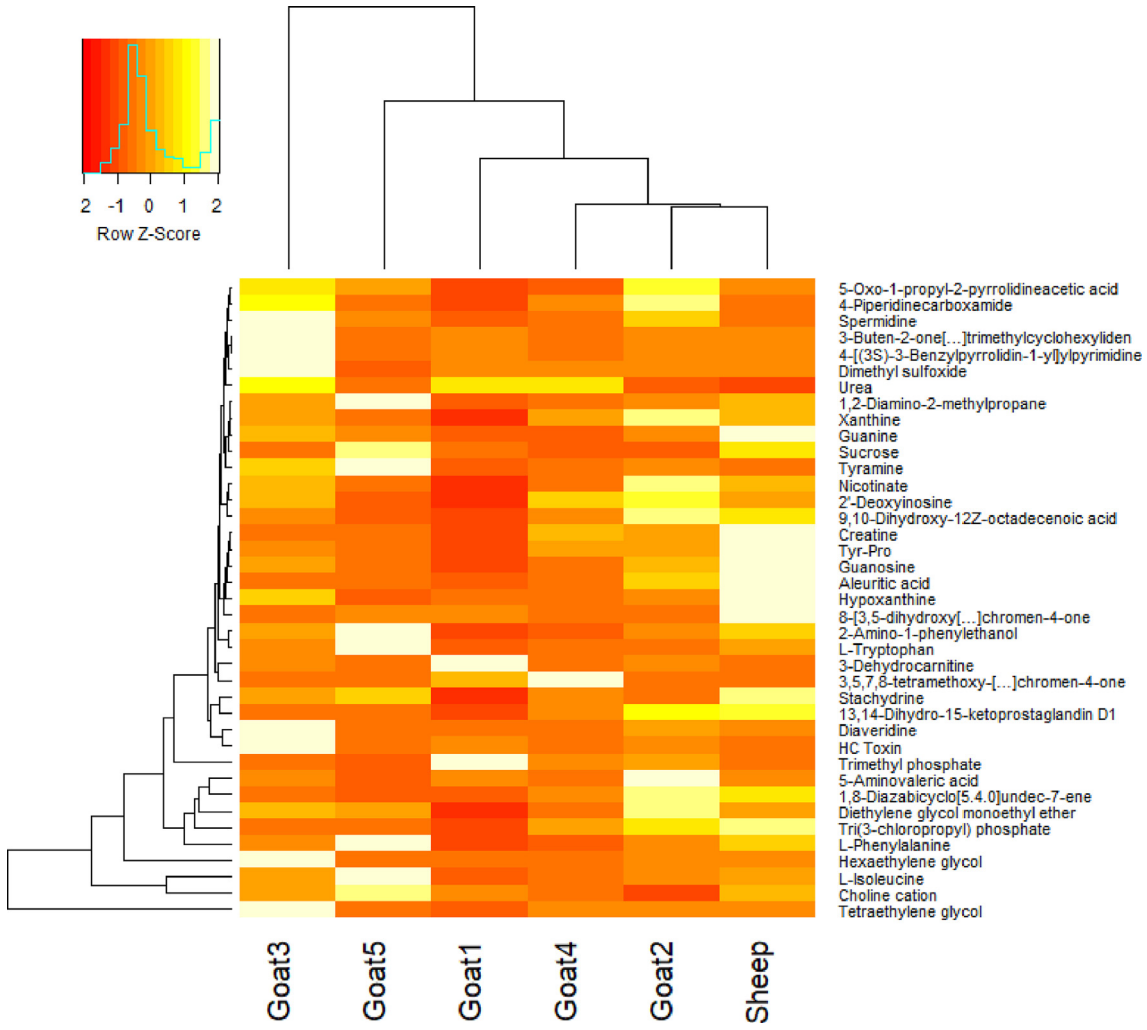

Fig. 3. Heatmap showing the abundance based on mTIC (sum peak height of all structurally annotated compounds) scores of the 39 detected metabolites found across the saliva samples (from Goats 1 to 5 and one sheep) used in the *in vitro* incubation.

Table 1
Effect of the incubation with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT), and sheep saliva (SHEEP) on *in vitro* rumen fermentation and the abundance of the major rumen microbial groups.

	Saliva				SEM	*P*-value
Item	AUT	OWN	GOAT	SHEEP		
pH	6.65^{a}	6.62^{a}	6.56^{b}	6.64^{a}	0.0087	<0.001
Gas Volume, ml/12 h	5.97^{b}	6.55^{a}	6.60^{a}	6.61^{a}	0.118	0.003
Total VFA, mM	58.8	60.1	61.3	62.4	0.786	0.287
Acetate, %	63.9	64.7	64.5	65.3	0.195	0.061
Propionate, %	22.7^{ab}	22.3^{b}	23.6^{a}	21.8^{b}	0.200	0.003
Isobutyrate, %	1.32	1.34	1.24	1.26	0.0389	0.766
Butyrate, %	8.52^{a}	8.06^{ab}	7.58^{b}	8.39^{a}	0.128	0.018
Isovalerate, %	1.67^{ab}	1.78^{a}	1.49^{c}	1.61^{bc}	0.0345	0.005
Valerate, %	1.62	1.66	1.54	1.60	0.0365	0.089
Ac/Pro	2.82^{b}	2.91^{ab}	2.74^{bc}	3.00^{a}	0.305	0.005
Microbes, log10 copy/ml						
Bacteria	10.2^{bc}	10.4^{ab}	10.2^{c}	10.4^{a}	0.0341	0.013
Archaea	7.09	7.11	7.00	7.23	0.0367	0.156
Protozoa	6.01^{ab}	6.17^{ab}	5.75^{b}	6.38^{a}	0.0924	0.046
Fungi	5.89	6.11	5.90	5.97	0.0697	0.297

Within a row, means with different letters differ ($P < 0.005$). VFAs = Volatile fatty acids. Ac/Pro = acetate/propionate ratio.

sequences. The prokaryotic alpha-diversity in terms of observed ASVs, Chao1, Shannon and Simpson indexes within the incubation bottles was not affected as a consequence of the incubation with the different types of saliva (Supplementary Fig. S1).

The analysis of the prokaryotic community based on the Venn diagram (Fig. 4) showed that a majority of the detected ASVs (959) were shared across the four saliva treatments. AUT was the treatment with the least overlapping ASVs with the rest of the

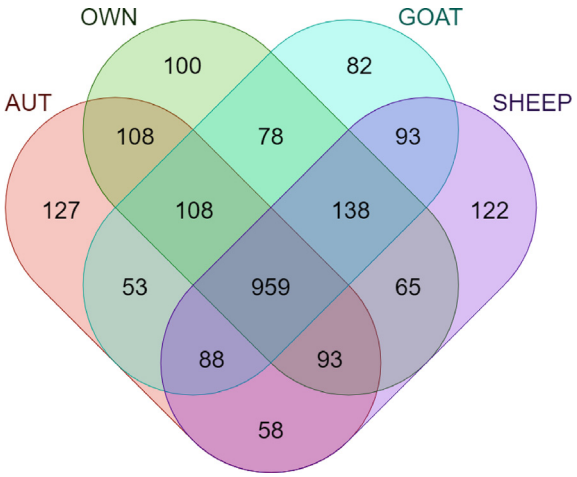

Fig. 4. Venn diagram showing the unique and overlapping prokaryotic ASVs (amplicon sequence variants) across the four saliva treatments used in the rumen incubation: autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT), and sheep saliva (SHEEP).

treatments (1 468 vs 1 549 vs 1 517 vs 1 494 in AUT, OWN, GOAT and SHEEP treatments, respectively), given its low degree of similarity with the other treatments regarding the prokaryotic community composition.

PERMANOVA analysis showed that the differences in the prokaryotic community structure across saliva treatments were significant in specific pair-wise comparisons (Fig. 5). The AUT saliva promoted the largest differences in the prokaryotic community structure across treatments. These differences were supported by the subsequent sPLS-DA plot, in which the component 1 axis (explaining 5% of the total variation) sorted the AUT samples apart from the rest, whereas the component 2 (explaining 4% of the total variation) disaggregated the OWN from the other two treatments with fresh saliva (GOAT and SHEEP). PERMANOVA analysis showed no significant differences in the prokaryotic community structure between OWN, GOAT and SHEEP treatments.

The relative abundance of the identified prokaryotic taxa was moderately variable according to the saliva treatment (Supplementary Table S2). At phyla level, four out of 18 had significantly different abundances across saliva treatments. *Actinobacteria* (average 2.73% relative abundance) was more predominant in bottles incubated with GOAT and SHEEP saliva (P = 0.0385). On the contrary, *Proteobacteria* (average 6.88%) was more predominant in bottles incubated with AUT and OWN saliva. Thirteen out of the 32 most abundant prokaryotic families (Fig. 6) and twelve out of the 33 most abundant genera denoted differences across the saliva treatments. *Prevotella 1*, the most abundant genus (22.4% sequences), was 22.3% more abundant in GOAT and SHEEP compared with AUT. Several relevant taxa including *Atopobium, Olsenella, Lachnospiraceae XPB1014 group* and *Streptococcus* also showed a greater abundance in GOAT samples, while Elusimicrobia and *Saccharimonadaceae* were more abundant in SHEEP. On the contrary, AUT samples had higher levels of *Bacteroides, Prevotellaceae UCG-003, Rikenellaceae, Family XIII, [Eubacterium] oxidoreducens group, Butyrivibrio, Succinivibrionaceae UCG-002* and *Veillonellaceae*. In OWN samples, only *F082, Prevotellaceae UCG-001, Quinella* and *Succinivibrionaceae* were more abundant compared to other treatments.

Effects of the type of saliva on the metabolomics profile and correlations with microbial taxa

The MS-MS metabolomics analysis on *in vitro* incubation samples identified 19 compounds after processing and filtration

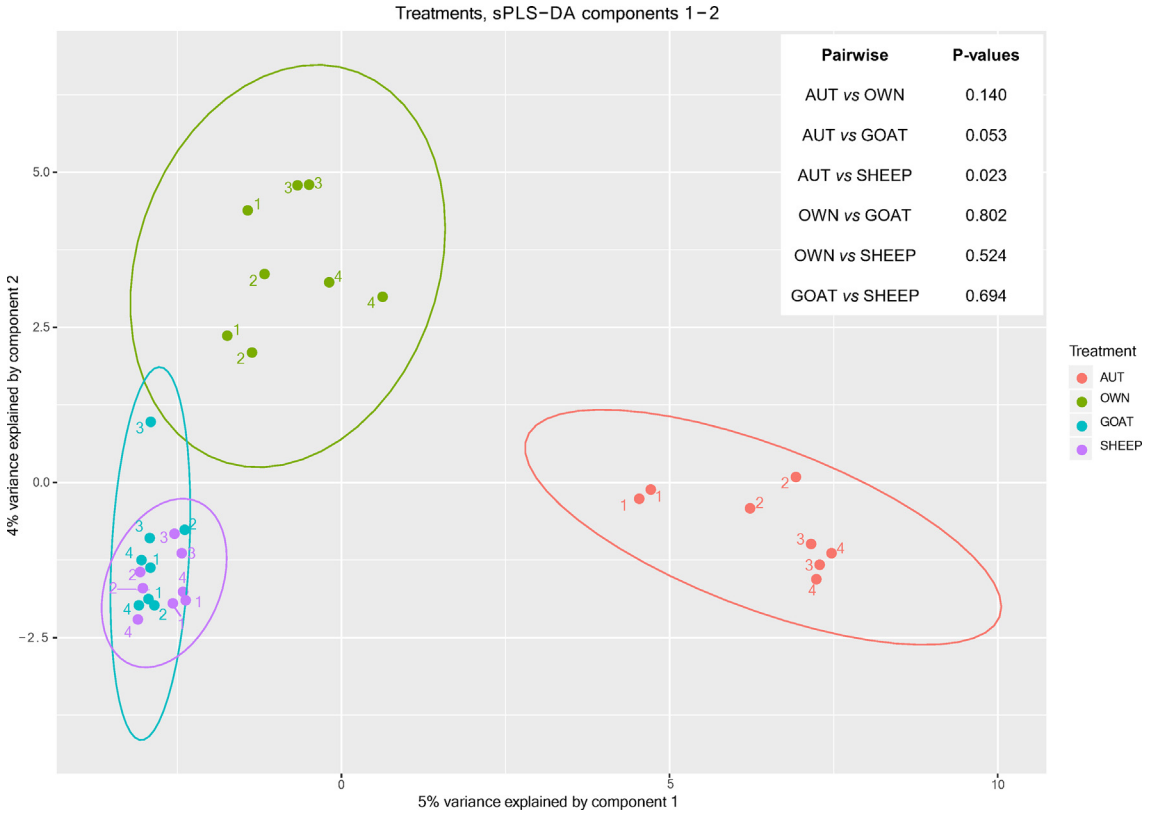

Fig. 5. Sparse partial least squares discriminant analysis (SPLS-DA) of the prokaryotic communities in rumen content incubated with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT), and sheep saliva (SHEEP). Numbers 1–4 in the plot account for the rumen fluid donors.

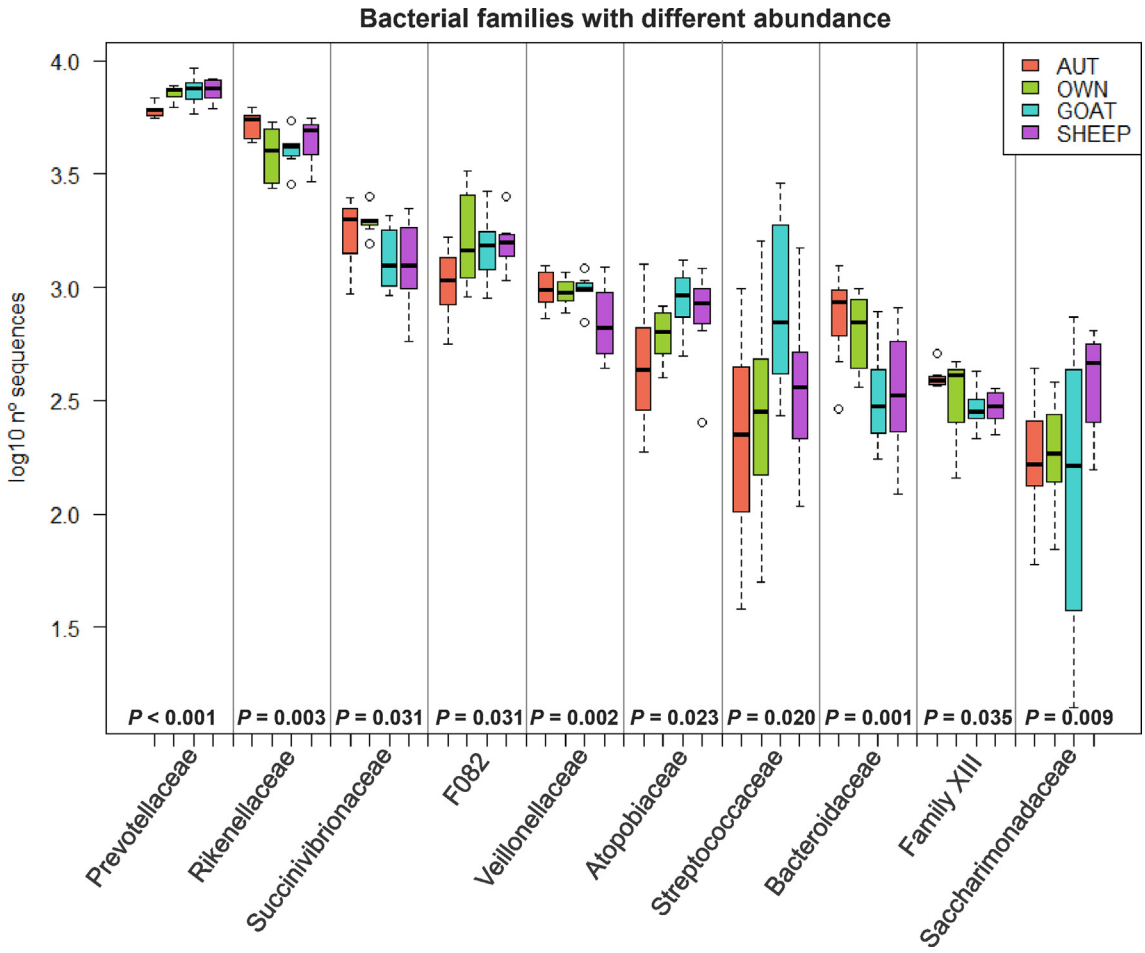

Fig. 6. Relative abundance of the ten most abundant prokaryotic families in rumen content incubated with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT) or sheep saliva (SHEEP).

(Fig. 7, Supplementary Table S3). On average, the most abundant metabolite was 15-Ketoprostaglandin E1, followed by ethyldiethanolamine and N-Methyl-2-pyrrolidone. Eight metabolites presented significantly different abundances according to saliva treatment (Supplementary Table S3). The heatmap based on mTIC values (Fig. 7) clustered samples from AUT treatment separately and then separated samples from SHEEP to those from GOAT and OWN treatments. This was further demonstrated, in agreement with microbial diversity results, by the significantly distinct metabolomic profile in AUT treatment compared with the other three treatments and that the SHEEP metabolome was different ($P < 0.005$) from that of the GOAT and OWN samples.

Spearman correlations (Supplementary Table S4) between microbial taxa and fermentation parameters and metabolome showed that a number of bacteria (*Tenericutes, Anaeroplasmataceae, Methanobacteraiceae* and *p-251-o5*) correlated positively with the production of VFA. On the other hand, butyrate was the VFA that showed the greatest number of negative correlations with microbial phyla and families. *Prevotellaceae*, the most abundant rumen bacterial family, showed strong correlations with a number of metabolites including Nicotinamide, Tryptamine and Tyramine.

Discussion

In our study, a thorough description of the protein and metabolite components of the different types of saliva was achieved prior to incubation with rumen fluid. The immunological profiling of fresh saliva samples from the five goats and one sheep revealed relatively low concentrations of IgA compared to the 5.95 mg/ml recently reported in bovine saliva (Fouhse et al., 2017). Previous works (Mach and Pahud, 1971; Lascelles and McDowell, 1974) reported higher IgA saliva concentrations (560 μg/ml, 157 μg/ml; respectively) than those observed in our study (37.6 μg/ml), whereas other studies found difficulties to quantify IgA in saliva (Porter and Noakes, 1970). As expected, the average concentration of IgA, which is the major immunoglobulin in ruminants' saliva (Lascelles and McDowell, 1974), was ~4-fold higher than that of IgG. Interestingly, even though this IgA:IgG ratio was maintained in the sheep's saliva, both concentrations were notably lower in comparison with the other saliva samples.

The total identified proteins across the saliva samples used in our study (195) were much greater than the 33 and 13 proteins annotated in sheep and goat saliva following a two-dimensional gel electrophoresis (2D PAGE) approach with two different spectrometry methods (Lamy et al., 2009, 2011; respectively). We hypothesise that this difference could partially be caused by the utilisation of a protein database such as TrEMBL which, unlike SwissProt, contains computationally annotated protein features instead of manually reviewed annotated proteins. A comprehensive study of the bovine salivary proteome where similar non-targeted MS-MS approaches were used (Ang et al., 2011) identified an average of 179 proteins across different sample preparation methods, which is similar to our figure and slightly closer to the

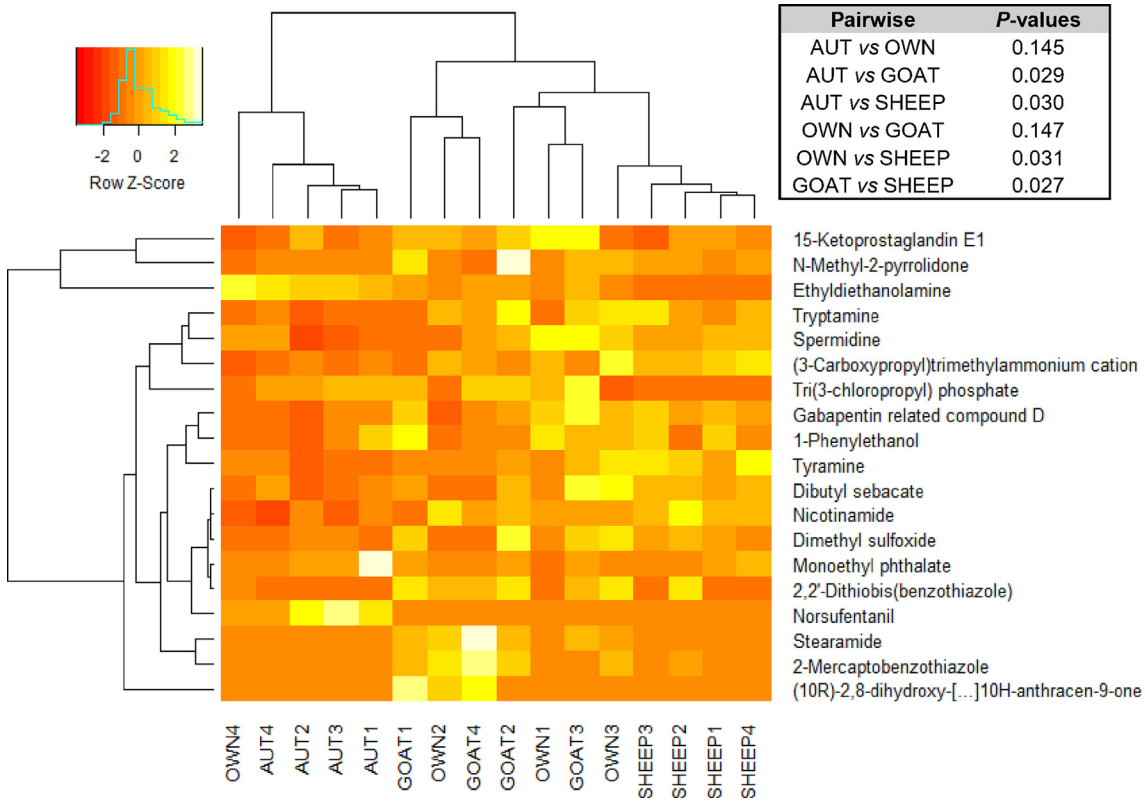

Fig. 7. Heatmap showing the abundance based on mTIC (sum peak height of all structurally annotated compounds) scores of the 19 detected metabolites found in rumen content incubated with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT) and sheep saliva (SHEEP).

hundreds of proteins identified in human saliva studies (Loo et al., 2010). Like in our study, variability based on different methodologies used and/or animal specificity in previous works played a significant role in this high rate of detected proteins. Such a wide array of salivary proteins are involved in numerous physiological functions across the animal kingdom (Mandel, 1987). Despite the inter- and intra-species variability with regard to salivary protein components in ruminants, these proteins seem to be involved in similar physiological functions (Ang et al., 2011). Indeed, the functional profile of the salivary proteins detected in cows (Ang et al., 2011) was pretty consistent with that found in goat and sheep proteins identified in our study. Most of them are involved in nutrient binding, transport, enzymatic activity and immune response. In our study, the importance of the saliva as part of the immune system is reflected by the fact that five out of the 30 most abundant salivary proteins (Lactoferrin Fragment, Cathelicidin-2, Lactoferrin, HSP27 protein, Cathelicidin-1 Fragment) are involved in microbial modulation (Fábián et al., 2012).

A previous batch culture *in vitro* study revealed that pre-incubation of specific diets (such as tannins-rich forages) with either sheep or goat saliva had a positive effect on diet degradation when incubated with rumen fluid (Ammar et al., 2013). Contrarily, other works have reported that the diet provided to ruminants and the saliva composition (including its protein fraction) have only minor effects on the rumen microbial activity (Ammar et al., 2011). Similarly, saliva composition and production were barely changed when different diets were provided to adult sheep (Salem et al., 2013). The lack of clear and consistent effects in these studies could be caused by the relatively short time of incubation (48 h), which might not be sufficient for the salivary components to effectively modulate microbial composition and activity. In this context, the use of different diets or the inoculation with unique microbial strains have been suggested to induce a number of immunological mechanisms in the gastrointestinal tract (**GIT**) (Yáñez-Ruiz et al., 2015). This has been reported to be of particular importance with regard to immunological proteins (mainly Ig), given that their concentration varies significantly depending on their rate of secretion through saliva (Subharat et al., 2016), which greatly depends on the presence of specific microorganisms in the rumen (Sharpe et al., 1977).

The metabolomics profile of the ruminants' saliva has not been thoroughly explored to date. In general, research on the saliva metabolome has been focused on the identification and characterisation of salivary biomarkers that could be used as indicators for the detection of a number of diseases (Yoshizawa et al., 2013). Other studies have attempted to better assess the metabolome composition throughout the gut, and the cross-effects that might take place between this and the host-microbiota (Gardner et al., 2019; Nicholson et al., 2012). In our study, substantial amounts of polyethylene glycol derivatives were detected, which could come from the use of commercial sponges for saliva collection. Overall, individual specificity on the saliva metabolome observed across our samples could most likely be driven by the unique microbiota present in each animal (Gardner et al., 2019) or by the different exposure to antigens, that altogether could be shaped by salivary proteins with immunological function (Palma-Hidalgo et al., 2021a). The substantial differences between GOAT and SHEEP saliva observed in the proteome and metabolome indicated a species-specificity in the abundance of salivary compounds which could partially explain the rumen microbial differences observed between these two species in previous works (Henderson et al., 2015; Langda et al., 2020).

Our semi-continuous incubation system showed a maximum microbial activity in the first hours of incubation and then progressively decreased, becoming stable in terms of pH and gas production from 48 hours and thereafter, indicating a stabilisation of

the microbial community. The analysis of the fermentation parameters after this adaptation to the *in vitro* conditions (days 6 and 7) showed that gas production was very low in AUT bottles compared with the rest, suggesting that untreated saliva from goats or sheep contain bioactive components that enhance fermentative activity. At this stage of incubation, the saliva donor species was the most influential factor *in vitro* fermentation as the SHEEP saliva promoted the highest levels of fermentative activity (+4% gas production) as well as the greatest bacterial and protozoal concentrations (+2 and +7%, respectively). The high butyrate molar proportion and acetate: propionate ratio in SHEEP samples also suggest that a greater fibrolytic activity could have taken place by the more abundant rumen protozoa present in this treatment (Belanche et al., 2019; Eugène et al., 2004). These differences in *in vitro* rumen fermentation when incubating with saliva from these two animal species were also reported by Ammar et al. (2013) using tannins-rich substrates, which again suggests that the unique salivary composition of each species or even individual animals may modulate microbial activity differently.

Despite that microbial diversity remained unchanged across treatments, incubation with different salivas had an effect on the microbial community structure, promoting the growth of different bacteria, which likely led to the changes in the fermentation profile as discussed above (Newbold and Ramos-Morales, 2020). Incubation with AUT saliva led to the most divergent rumen microbial community in terms of overlapping ASVs with other treatments and general microbial composition. At phyla level, the relative abundance of the two main bacteria phyla across all treatments was 53% for *Bacteroidetes* and 30% for *Firmicutes*, a ratio (1.76) which is almost half (3.25) of what has been previously described in the rumen of goats (Palma-Hidalgo et al., 2021c). We hypothesise that the salivary proteins promoted the growth of *Firmicutes* bacteria, which have been demonstrated to be more abundant in the proximal GIT or the oral cavity (Fouhse et al., 2017; Yeoman et al., 2018). The salivary components of GOAT and SHEEP treatments also increased the proliferation of *Actinobacteria* (Fouhse et al., 2017) which includes numerous species known for their ability to degrade complex compounds and fibre (Barka et al., 2016).

The three microbial taxa that contributed the most to make the AUT prokaryotic community differ from the rest (particularly that from SHEEP) were *Proteobacteria* phylum and *Prevotellaceae and Rikenellaceae* families. With the exception of the AUT-abundant *Succinivibrionaceae* family, which has been recently correlated with animal growth and VFA production (Palma-Hidalgo et al., 2021c), *Proteobacteria* are commonly categorised as early rumen colonisers (Jami et al., 2013) and have been often associated with a suboptimal rumen microbial development. The greater abundance of this phylum in AUT treatment may suggest a deficient regulation by the lack of salivary bioactive components with immunological function, which were denatured by autoclaving treatment (Palma-Hidalgo et al., 2021a). This explanation would also be in line with the lower abundances in AUT samples of *Prevotella 1* and *Prevotellaceae* (−22.3%), which is a cornerstone bacterial genus in the rumen and ruminant's oral cavity (Rey et al., 2014; Tapio et al., 2016) and plays a pivotal role in the rumen metabolism (Precup and Vodnar, 2019). Given the positive symbiotic effects of most *Prevotella* species in the rumen, it might be possible that its growth could be (directly or indirectly) stimulated by its incubation with salivary proteins (i.e. immunoglobulins), as it has been demonstrated with other commensal bacteria in mice (Donaldson et al., 2018; Peterson et al., 2007). Indeed, IgA and IgG and its different isoforms have been shown to modulate bacterial populations throughout the GIT (Tsuruta et al., 2012) to maintain mucosal homeostasis (Mantis et al., 2011). However, IgA-tagged bovine oral or rumen microbiota have been reported to include a significant lower abundance of *Prevotellaceae* compared to regular rumen microbiota (Fouhse et al., 2017). The high variability in the Ig concentrations in our study, and particularly, the low concentrations (−34% IgA) in the sheep saliva coupled with the high abundances of *Prevotellaceae* in the SHEEP treatment, suggest that other immunological mechanisms driven by different proteins or molecules (*e.g.* cytokines, defensins, cathelicidins, miRNA; Yáñez-Ruiz et al., 2015) could also be involved in modulation of the rumen microbiome and their fermentative activity (Palma-Hidalgo et al., 2021a). The specificity of these modulatory mechanisms, which seems to vary moderately across species and individuals, may be partially responsible of the resilience and individual host specificity of the ruminal microbiota reported through complete rumen exchange experiments (Weimer, 2015). In line with this, our results suggest that the bioactive components of saliva have a positive effect on the proliferation of crucial goat rumen bacteria as well as on the microbiota capable of degrading solid feeds. However, these positive effects on rumen microbial composition and activity are not as clear when goat rumen fluid is incubated with the specific salivary components of the same animal (OWN), indicating that the influx of new exogenous salivary elements could have synergistic effects on the rumen microbiome and fermentation. The different effects on the rumen microbial composition and activity seemed to be more notable across different species, which supports the fact that the specificity of the goat's or sheep's saliva composition (Lamy et al., 2009) observed in our study leads to the development of distinct microbial communities under similar dietary conditions (Langda et al., 2020).

Previous research on the rumen metabolome (Artegoitia et al., 2017; de Almeida et al., 2018) also resulted in the detection of thousands of distinct 'raw' metabolic features. However, after quality filtering, the vast majority of them could not be reliably annotated using different search engines and libraries, which highlights the great complexity of the rumen metabolome and its potential to act as a reservoir of novel compounds. The amount of annotated metabolites (19) compared with the 67 identified by de Almeida et al. (2018) made it difficult to discern clear effects of the incubation with different salivas on the rumen metabolomics profile. Despite this, the variability in the level of detection of the different metabolites indicate how its presence and abundance are most likely driven by salivary components *per se* or by the distinct microbial community (Gardner et al., 2019) modulated by saliva from different donors. The distinct salivary components across our treatments seem to have a strong influence on the rumen metabolome as this varies depending on whether they are constituents of goat's or sheep's saliva. Our data suggest that saliva components may partly be responsible for the host species-specific rumen microbiota and related metabolites, potentially due to the co-evolution of the microbiome and host (Koskella and Bergelson, 2020). Moreover, our study showed that AUT saliva promoted different metabolic pathways than untreated saliva, indicating the presence of salivary metabolites with bioactive properties able to modulate the rumen microbiota and nutrient metabolism. Furthermore, the correlations analysis showed that specific microbial taxa contribute differently to the relative abundance of certain metabolites *in vitro*, thus highlighting the importance of salivary elements modulating the microbial community, which at the same time has an important effect on the rumen metabolism. The limited amount of detected metabolites did not allow to draw clear conclusions on the relationships between specific microorganisms and the metabolome resulting from their activity.

Incubation of rumen fluid with mineral buffer instead of saliva has been shown to decrease the fermentative activity in a recent study (Palma-Hidalgo et al., 2021a). The inclusion of such negative control treatment could have also enabled further insights into the role of the inorganic components of saliva (present in AUT) on the

rumen microbial composition as well as the resulting metabolome. Hence, further studies are needed to identify the potential implications of these salivary modulatory effects on the rumen health and productive outcomes.

Conclusions

The characterisation of sheep and goats saliva showed distinct metabolomic and proteomic profiles across individuals and animal species, though the general functions (enzymatic, transport, immune response) remain consistent. Inactivating these compounds (i.e. autoclaving) exhibits an important change in the function of saliva in shaping rumen fermentation and microbial community. This finding together with the differences observed between species suggests that the cross-talk mechanisms between salivary components and rumen microbiota can be specific for individuals and/or animal species and that may contribute to the host selection of the commensal microbiota and its function. If these findings are confirmed *in vivo*, they could help to better understand host-microbiota cross-talk and open the possibility of developing new strategies to modulate the rumen function and activity.

Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.animal.2023.100895.

Ethics approval

Experimental protocols were approved by the Ethical Committee for Animal Research at EEZ-CSIC and animal procedures were conducted by trained personnel according to the Spanish Animal Experimentation guidelines (RD 53/2013).

Data and model availability statement

Raw sequences read data from the 16S sequencing analysis were deposited at the European Nucleotide Archive repository (accession: PRJEB45956; https://www.ebi.ac.uk/ena/browser/view/PRJEB45956). The data that support the study findings are available to reviewers, or available from the authors upon request.

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Declaration of interest

The authors declare no conflict of interest.

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