

# Multi-Omics Study of The Salivary Modulation of The Rumen Microbiome

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## Research Article

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# Abstract

Ruminants are able to produce large quantities of saliva which enter into the rumen. 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. A total of 16 semi-continuous *in vitro* cultures were used to incubate rumen fluid from 4 donor goats inoculated with autoclaved saliva (AUT) as negative control, saliva from the same rumen fluid donor (OWN) as positive control, and either GOAT or SHEEP saliva as experimental interventions. Fermentation was monitored throughout the 7 days of incubation and the prokaryotic communities and metabolome were analysed at day 7 of incubation. Characterization of the salivas used prior to incubation showed a high degree of individual variability in terms of the salivary metabolites and proteins, including immunoglobulins. The prokaryotic community composition in AUT incubators was the most divergent across treatments, suggesting a modulatory effect of active salivary components, which were not affected in the other treatments (OWN, GOAT and SHEEP). The differences across treatments in microbial diversity were mostly caused by a greater abundance of *Proteobacteria* and *Rikenellaceae* and lower of *Prevotellaceae*, a key rumen bacterium with greater abundance in GOAT and SHEEP treatments. These results suggest that specific salivary components contribute to host-associated role in selecting the rumen commensal microbiota and its activity.

# Introduction

The rumen of ruminant animals contains a great diversity of prokaryotic (bacteria, archaea, virus) and eukaryotic (protozoa and fungi) micro-organisms 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 utilization of feeds and the environmental impact through methane emissions (Jami et al., 2014). The digestive microbiomes in most mammals are controlled by host genetic variation (Koskella & Bergelson, 2020) through multiple processes, one of the most crucial being immune modulation, by secreting many substances from epithelial cells (i.e. antimicrobial peptides, immunoglobulins,..) and germline-encoded pattern recognition receptors (Zheng et al., 2020). However, in the rumen no organized lymphoid tissue exists in the epithelium (Sharpe et al., 1977), which includes up to a 15 cell layer, that limit the permeability of large molecules. Saliva has been suggested as 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. Saliva constituents include a significant amount of ions (mainly bicarbonate and phosphate), that help maintain rumen osmotic pressure and pH within physiological range (Warner & 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 & 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), modulate 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 & 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 diversity using a 7-days semi-continuous *in vitro* trial. A detailed characterization 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, goat-saliva, sheep-saliva and autoclaved-saliva) to elucidate the potential modulatory effect on the rumen microbial ecosystem.

## Results

# Immunological, proteomic and metabolomic profiling of the individual salivas

Saliva from Goats 1-4 (used in OWN treatment), Goat 5 (used in GOAT treatment) and the sheep (used in SHEEP treatment) showed distinct immunological, proteomic and metabolomic profiles. Average protein concentration across salivas was  $908 \pm 146$   $\mu\text{g/ml}$ . IgA Elisa resulted in an IgA concentration of 36.6, 30.0, 26.3 and 57.3  $\mu\text{g/ml}$  in Goats 1-4, respectively, in comparison with 44.6  $\mu\text{g/ml}$  (+19%) in Goat 5's saliva and 29.1  $\mu\text{g/ml}$  (-22%) in the sheep's saliva. In the case of salivary IgG quantification, the concentration in Goats 1-4 was 9.98, 9.72, 11.7 and 11.5  $\mu\text{g/ml}$ , respectively; similar to that in Goat 5's saliva (10.48  $\mu\text{g/ml}$ ), but much higher (+24%) than that of the sheep's saliva (8.12  $\mu\text{g/ml}$ ).

The proteomic MS/MS analysis of the saliva samples resulted in the identification of 195 proteins/polypeptides across the 6 samples. The average number of proteins per sample was 59, with Goat 5's saliva having the lowest count (46), and the sheep's 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 (Figure 1). 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 hemoglobin 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 6 samples. Interestingly, not the sheep's but Goat 3's saliva

turned out to be, compared with the others, the most different sample with regards to the proteomic profile. However, unlike all the goats' salivas, sheep's saliva did not contain goat-specific *Capra hircus* Akirin 2 mRNA but it did have high values of other proteins such as Glutathione S-transferase and Insulin-like growth factor 1, which were almost not present in goat salivas.

The metabolomic MS/MS 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 (Figure 2). The peak heights of most of the compounds detected in the MS/MS 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's saliva was, again, the one with the most different metabolomic profile. On average, the 1st (Tetraethylene glycol), 4th (Hexaethylene glycol) and 5th (Diethylene glycol monoethyl ether) most abundant metabolites 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 1000 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 acids 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 1**). The incubation of rumen fluid from 4 different goats with AUT, OWN, GOAT and SHEEP salivas led to substantial differences in the fermentation pattern (Table 1). Incubation with AUT led to 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) compared with the rest.

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	<i>P</i> -value
	AUT	OWN	GOAT	SHEEP		
pH	6.65 <sup>a</sup>	6.62 <sup>a</sup>	6.56 <sup>b</sup>	6.64 <sup>a</sup>	0.0087	<0.001
Gas Volume, ml/12h	5.97 <sup>b</sup>	6.55 <sup>a</sup>	6.60 <sup>a</sup>	6.61 <sup>a</sup>	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 <sup>ab</sup>	22.3 <sup>b</sup>	23.6 <sup>a</sup>	21.8 <sup>b</sup>	0.200	0.003
Isobutyrate, %	1.32	1.34	1.24	1.26	0.0389	0.766
Butyrate, %	8.52 <sup>a</sup>	8.06 <sup>ab</sup>	7.58 <sup>b</sup>	8.39 <sup>a</sup>	0.128	0.018
Isovalerate, %	1.67 <sup>ab</sup>	1.78 <sup>a</sup>	1.49 <sup>c</sup>	1.61 <sup>bc</sup>	0.0345	0.005
Valerate, %	1.62	1.66	1.54	1.60	0.0365	0.089
Ac/Pro	2.82 <sup>b</sup>	2.91 <sup>ab</sup>	2.74 <sup>bc</sup>	3.00 <sup>a</sup>	0.305	0.005
Microbes, log <sub>10</sub> copy/ml						
Bacteria	10.2 <sup>bc</sup>	10.4 <sup>ab</sup>	10.2 <sup>c</sup>	10.4 <sup>a</sup>	0.0341	0.013
Archaea	7.09	7.11	7.00	7.23	0.0367	0.156
Protozoa	6.01 <sup>ab</sup>	6.17 <sup>ab</sup>	5.75 <sup>b</sup>	6.38 <sup>a</sup>	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 ( <i>P</i> < 0.05)						

Results from qPCR analyses showed significantly higher concentration of bacteria in bottles incubated with SHEEP and OWN salivas, whereas the lowest bacterial concentration was found when bottles were incubated with AUT and GOAT saliva (*P* = 0.013) (Table 1). Likewise, the abundance of rumen protozoa was the highest in SHEEP and the lowest in GOAT bottles (*P* = 0.046). No significant effects were noted on the methanogenic archaea or anaerobic fungi concentrations according to the different type of saliva.

## Microbial diversity

The sequencing analysis performed on incubation samples generated 41,514 ± 13,383 high quality prokaryotic sequences per sample. The number of sequences was normalized to 28,131 for further

processing and analyses. Good's coverage index was 98.8% on average and similar for the 4 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 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 Figure 1**).

The Venn diagram (Figure 3) showed that a majority of the detected ASVs (959) were shared across the 4 saliva treatments. AUT was the treatment with the least overlapping ASVs with the rest of the treatments (1468 vs. 1549 vs. 1517 vs. 1494 in AUT, OWN, GOAT and SHEEP treatments, respectively).

PERMANOVA analysis showed that the differences in the prokaryotic community structure across saliva treatments were significant in specific pair-wise comparisons (Figure 4). The clearest difference was that observed between the communities in the AUT and the rest of the treatments. The level of dissimilarity between the AUT community structure and in the other three treatments (mainly SHEEP and GOAT) was also very apparent in the subsequent sPLS-DA. The component 1 axis in the sPLS-DA (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 2**). At phyla level, 4 out of 18 had significantly different abundances across saliva treatments. *Actinobacteria* (2.73% average relative abundance) was more predominant in bottles incubated with GOAT and SHEEP saliva ( $P = 0.0385$ ). On the contrary, *Proteobacteria* (6.88% average relative abundance) was more predominant in bottles incubated with AUT and OWN saliva. Thirteen out of the 32 most abundant prokaryotic families (Figure 5) 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 metabolomic composition

The MS-MS metabolomics analysis on in vitro incubation samples identified 19 compounds after processing and filtration (Figure 6, **Supplementary Table 3**). 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. The heatmap based on mTIC values (Figure 6) clustered samples from AUT treatment separately and then separated samples from SHEEP to those from GOAT/OWN saliva 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.

## Discussion

In our study, a thorough description of the protein and metabolite components of the animals' saliva was achieved prior to incubation with rumen fluid. The immunological profiling of fresh saliva 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). Even though some previous works where ELISA was not used for quantification (Porter & Noakes, 1970) had difficulties at detecting IgA even after a 20 fold concentration using dialysis, others such as Mach & Pahud (1971) and Lascelles & McDowell (1974) reported much higher IgA saliva concentrations (560  $\mu\text{g/ml}$ , 157  $\mu\text{g/ml}$ ; respectively) than what we detected in our study (37.6  $\mu\text{g/ml}$ ). As expected, the average concentration of IgA, which is the major immunoglobulin in ruminants' saliva (Lascelles & McDowell, 1974), was  $\sim 4$  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 salivas.

The total identified proteins across the salivas used in our study (195) was 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). Despite the number of annotated proteins was much higher in our study, we hypothesize that this difference could partially be caused by the utilization 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 nontargeted 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 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 intraspecies variability with regards 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 which are involved in nutrient-binding, transport, enzymatic activity and, to a less extent, immune response.

A previous *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). On the other hand, other works have reported that the diet provided to ruminants and their saliva composition (including its protein fraction) have only minor effects on the rumen microbial activity (Ammar et al., 2011) and *vice-versa* (Salem et al., 2013). The lack of substantial effects found in these studies could be caused by the relatively short time of incubation (48 h) but also due to a missing exploration of the salivary proteome and metabolome, which we addressed in our study. 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 GIT (Yáñez-Ruiz et al., 2015). This has been reported to be of particular importance with regards 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 metabolomic 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 characterization 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 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), that altogether could be shaped by salivary proteins with immunological function (Palma-Hidalgo et al., 2021a). Moreover, 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 reached a peak of microbial activity in the first hours of incubation and then remained stable in terms of pH and gas production from 36 hours and thereafter. Through the last days of incubation, gas production was very low in AUT bottles compared with the rest, indicating 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 of the two small ruminants species were also reported by Ammar et al.

(2013) when using tannins-rich substrates, which again suggests that the unique salivary composition of each species or even individuals may modulate microbial activity differently.

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., 2021b). We hypothesize 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 salivas also increased the proliferation of saliva-abundant *Actinobacteria* (Fouhse et al., 2017) which includes numerous species known for their ability to degrade complex compounds like fiber (Barka et al., 2016).

The three microbial taxa that contributed the most to make the AUT prokaryotic composition 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., 2021b), *Proteobacteria* are commonly categorized as early rumen colonizers (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 most likely denatured by autoclaving (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 & Vodnar, 2019). Given the harmless commensal nature of most *Prevotella* species in the rumen, it might be possible that its growth could be (directly or indirectly) stimulated when incubating with untreated salivas by modulation of salivary protein components, namely 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 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 the stimulation or inhibition of the rumen microbes 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 fibrous 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. The fact that, after quality filtering, the vast majority of them could not be reliably annotated using different search engines and libraries speaks for the great complexity of the rumen metabolome and how it could be 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 of different species seem to have a strongest influence on the rumen content as they shape the rumen metabolome differently depending on whether they are constituents of goat's or sheep's saliva. Our data indicate that saliva components may partly responsible for the host species-specific rumen microbiota and related metabolites, potentially due to the co-evolution of the microbiome and host (Koskella & Bergelson, 2020).

The characterization 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) exhibit an important change in the function of saliva in shaping rumen fermentation and microbial community. This finding together with the differences observed between species suggest that the cross-talk mechanisms between salivary components and rumen microbiota can be specific for individuals and/or species and that may contribute to the host selection of the commensal microbiota and its function.

## 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 adult rumen-cannulated goats (Goat 1 to Goat 5) and one adult sheep, all housed in different pens, were used as saliva donors. During the study, all animals were fed at maintenance level with a diet consisting on 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  $190\times g$  for 10 min, then filtrated through 0.25  $\mu\text{m}$  pore size to remove microorganisms and large particles, pooled per animal and stored in aliquots at  $-80^{\circ}\text{C}$  until the start of the *in vitro* incubation. Additionally, equal volumes of saliva from goats 1-4 were mixed, autoclaved at  $121^{\circ}\text{C}$  for 30 min and stored at  $-80^{\circ}\text{C}$  (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

An *in vitro* semi-continuous incubation was conducted during 7 days using rumen fluid from 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 was incubated with 4 different types of saliva ( $n=4$ ): saliva from the same rumen fluid donor (Goats 1-4) (OWN) as positive control, saliva from goat 5 (GOAT), saliva from sheep (SHEEP) and pooled autoclaved saliva to denature active metabolites in the saliva but keeping the minerals, (AUT) from goats 1-4, as negative control. Incubations consisted in a total volume of 20 ml per bottle composed 6.67 mL of rumen fluid, 6.67 mL of saliva and 6.67 mL of bicarbonate buffer (3.5 g  $\text{NaHCO}_3$  + 0.4 g  $(\text{NH}_4)\text{HCO}_3$  in 100 ml  $\text{dH}_2\text{O}$ ). The same oats hay and commercial concentrate that were offered to the animals were grinded to 1mm size particles and used as incubation substrate (100 mg each).

In order to maintain an active *in vitro* system, every 12 hours (9.00 and 21.00), 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 homogenized 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, and 1/3 of diet (33 mg oats hay and 33 mg commercial concentrate). A continuous flow of  $\text{CO}_2$  was applied to each bottle through this process to maintain the anaerobic conditions. One sub-sample (800  $\mu\text{l}$ ) of the removed incubation content was taken at 12, 36, 60, 84, 108, 132 and 156 hours, mixed with 800  $\mu\text{L}$  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 acids (VFA) determination by gas chromatography (AutoSystem gas chromatograph, Perkin Elmer, Waltham, MA). A second sub-sample (200  $\mu\text{l}$ ) of the removed incubation content at day 7 was used for metabolome analysis following a similar procedure to that described for saliva samples. A third sub-sample (200  $\mu\text{l}$ ) taken at days 2, 4, 6 and 7 was used for DNA extraction using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Ltd., Barcelona, Spain).

## Characterization 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 (Pierce™ 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 nLC (Easy nano Liquid Chromatograph, Proxeon, Odense, 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 & 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 a 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). MS/MS spectra were processed and filtrated using MS-DIAL v 4.12 software (<http://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 normalized for each sample to allow comparisons across salivas.

## qPCR and next generation sequencing

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 (qPCR) using a iQ5 multicolor Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA, USA). Specific primers for the 16S bacterial rRNA gene (Maeda et al., 2003), *mcrA* gene for methanogenic archaea (Denman et al., 2007) and 18S rRNA genes for protozoa and anaerobic fungi (Sylvester et al., 2004 and Denman & McSweeney, 2006; respectively). Quantitative PCR standards consisted of the plasmid PCR 4-TOP (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 at 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 Miseq V3 (600 cycles) kit (Illumina Inc., San Diego, CA, USA). Primers used for the amplification were 5'-CCTACGGGNBGCASCAG-3' and reverse: 5'-GACTACNVGGGTATCTAATCC-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 (ASV) 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 99% reference sequences (Quast et al, 2013). Once alignment was performed, the number of sequences per sample for each microbial group was normalized across all the samples and singletons were removed. Raw sequences reads were deposited at European Nucleotide Archive repository (accession: PRJEB45956).

## 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:

$$Y_{ijklm} = \mu + S_i + T_j + CT_{ij} + R_k + e_{ijkl}$$

where  $Y_{ijklm}$  is the dependent, continuous variable,  $\mu$  is the overall population of the mean,  $S_i$  is the fixed effect the type of saliva ( $i$ = AUT vs OWN vs GOAT vs SHEEP),  $T_j$  is the fixed effect of the time ( $j$ = 12h vs 36h vs 60h vs 84h vs 108h vs 132h vs 156h sampling times),  $CT_{ij}$  is the interaction term,  $R_k$  is the random effect of the rumen fluid and  $e_{ijkl}$  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 log<sub>10</sub> transformed before the analysis to achieve a normal distribution. Only prokaryotic families & genera with relative abundance > 0.1% across saliva treatments were further considered for taxonomic analyses (in % of sequences). In all analyses, significant effects were declared at  $P < 0.05$  and tendency to difference at  $P < 0.1$ .

Proteomic and metabolomic heatmaps based on emPAI and mTIC values, respectively, were constructed using RStudio (R Foundation for Statistical Computing, Vienna, Austria) to characterize the salivas before incubation and the effect of saliva on the rumen metabolome at 156h 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 using a multiple list comparator

([www.molbiotools.com](http://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 log<sub>10</sub> transformed sequencing data with 999 random permutations. 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.

## Declarations

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### Author contributions

DRYR, CJN & AB: Conceptualization, validation and supervision.

JMPH, AB, JMPH, EJ & AIMG: Methodology, investigation, resources and formal analysis.

JMPH, AB, SED: Data curation and software.

DRYR: funding acquisition.

JMPH: Writing original draft.

DRYR, AB, SED & CJN: Writing – review and editing.

### Competing interests

The authors declare no competing interests.

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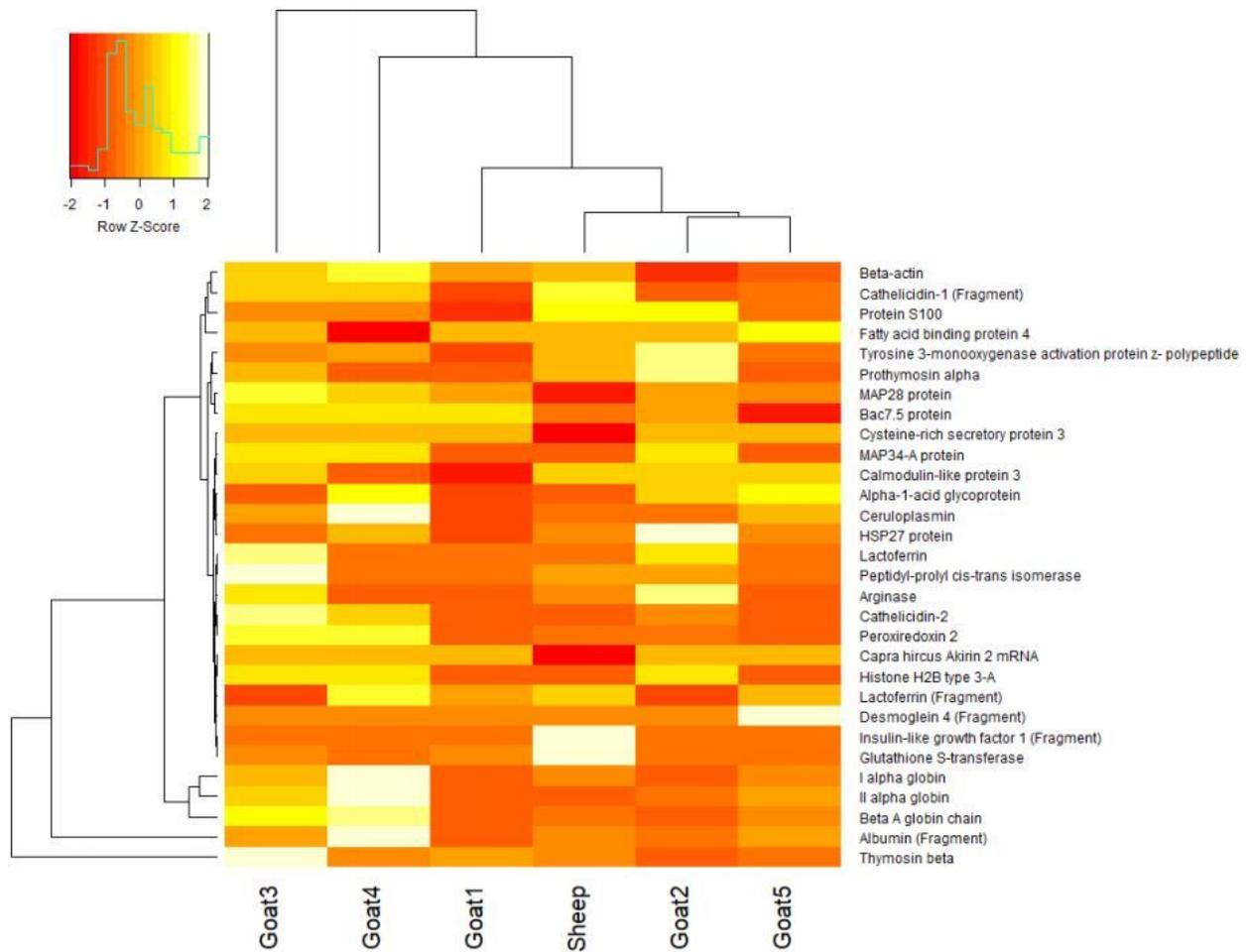
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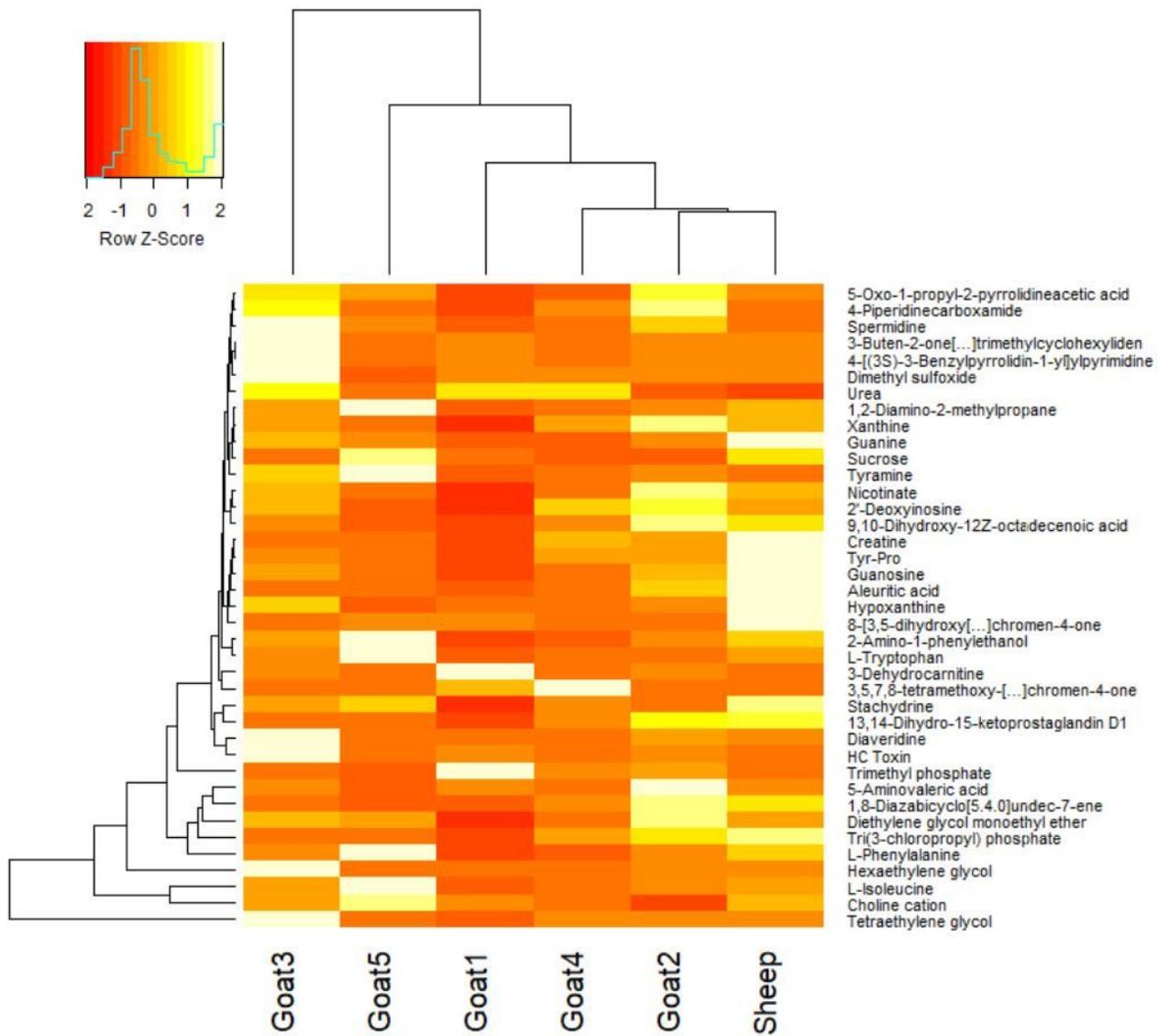
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## Figures



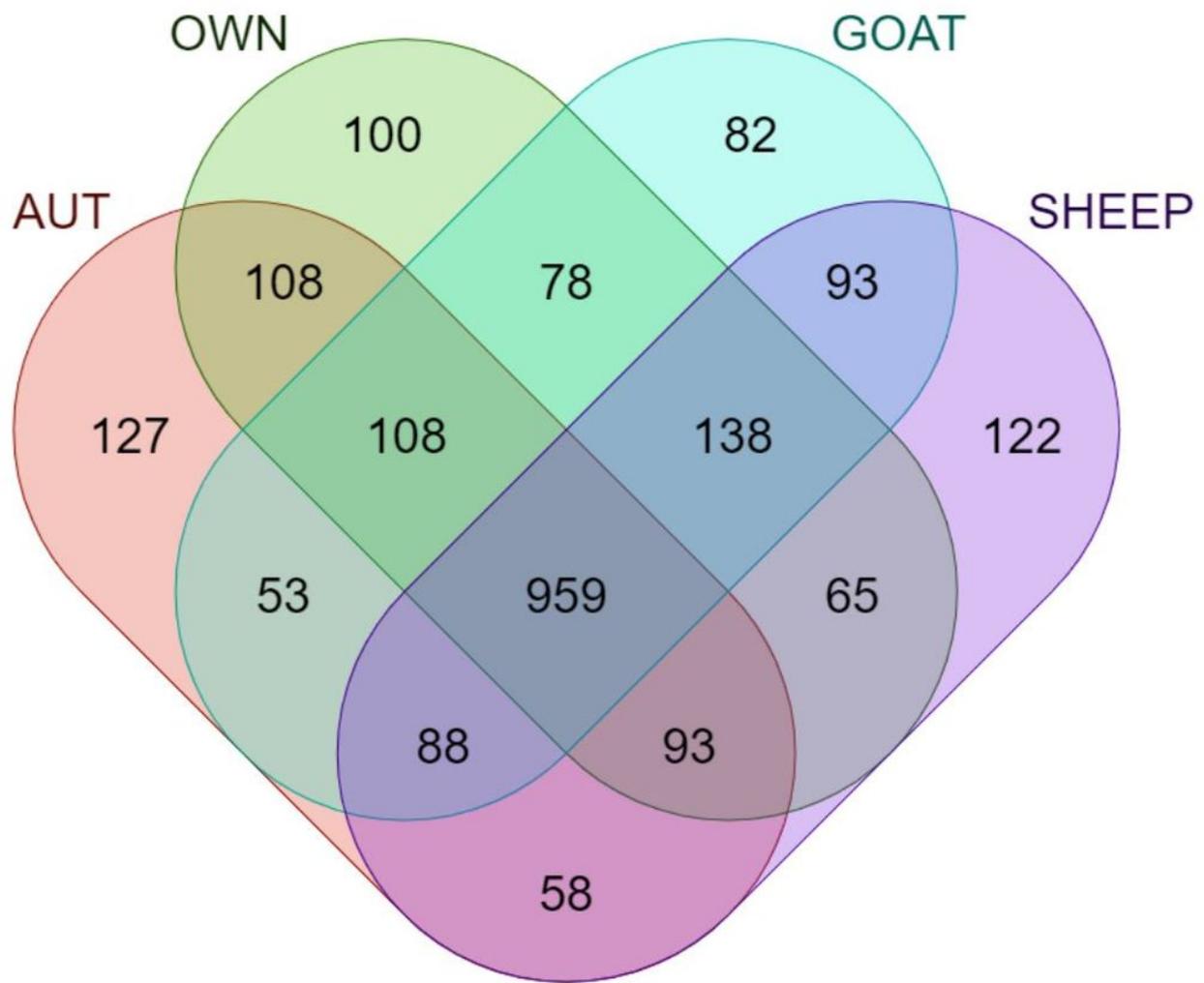
**Figure 1**

Heatmap showing the abundance based on emPAI values of the 30 most abundant proteins/polypeptides found across the salivas used for the in vitro incubation.



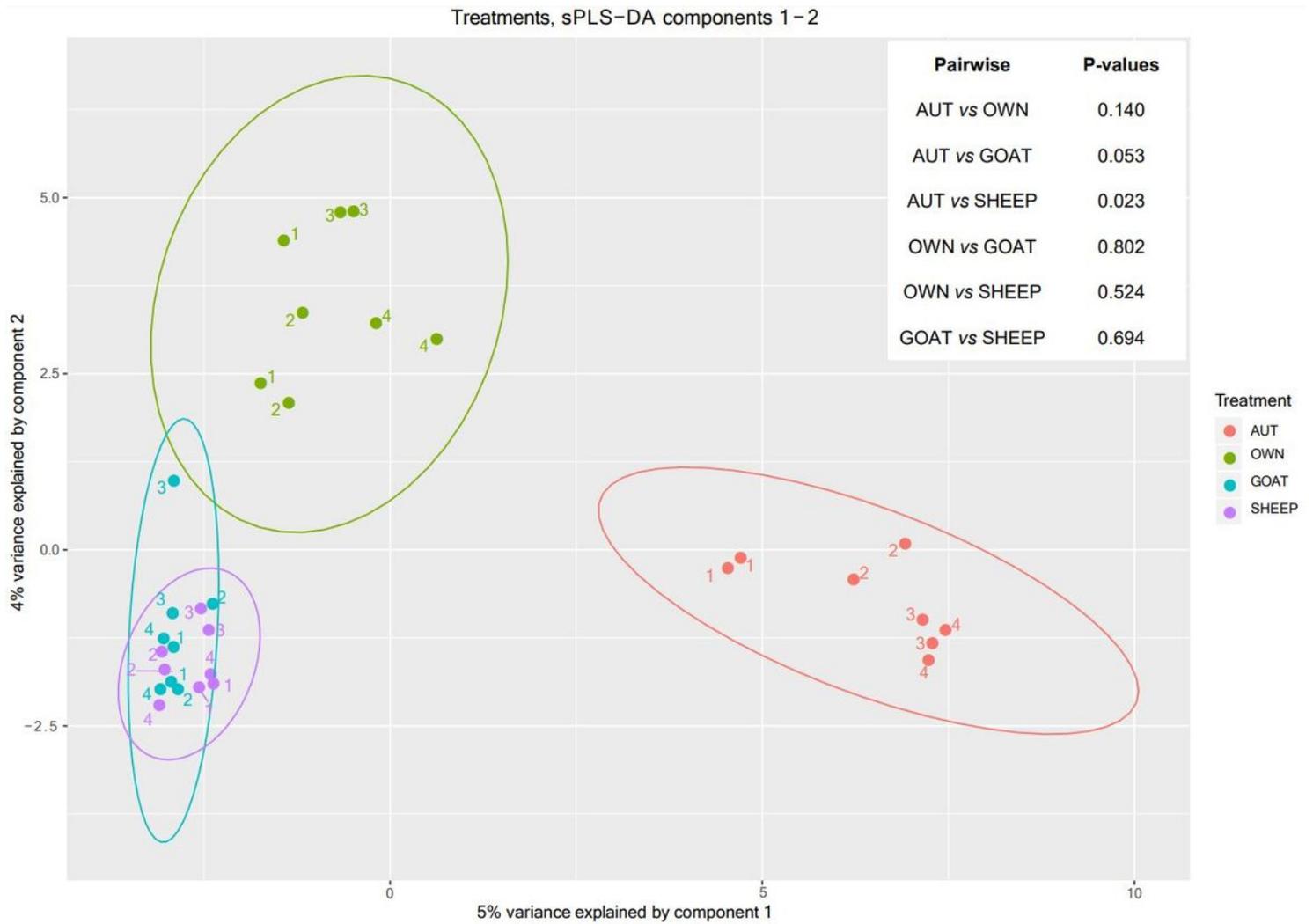
**Figure 2**

Heatmap showing the abundance based on mTIC scores of the 39 detected metabolites found across all saliva samples used in the in vitro incubation.



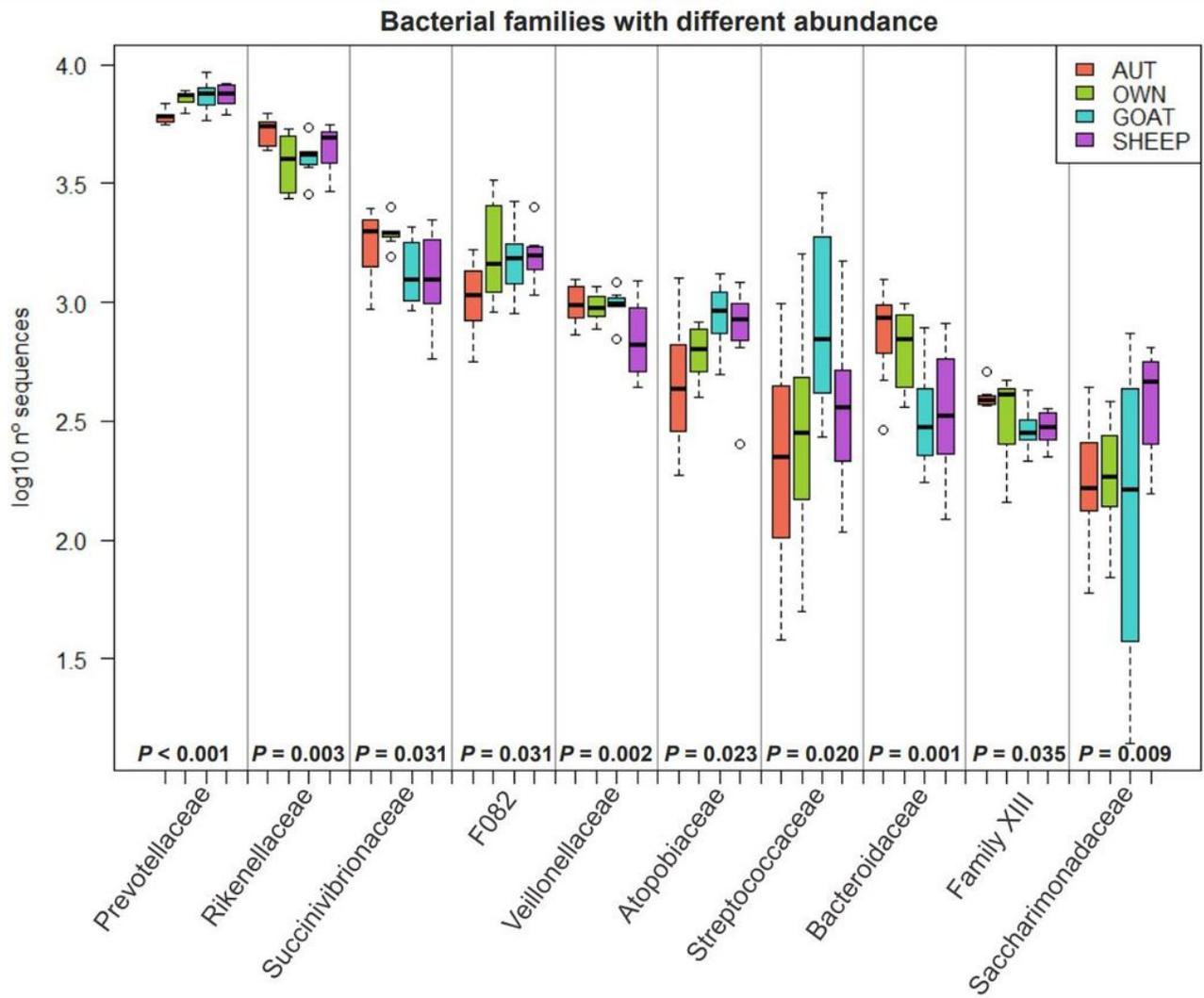
**Figure 3**

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



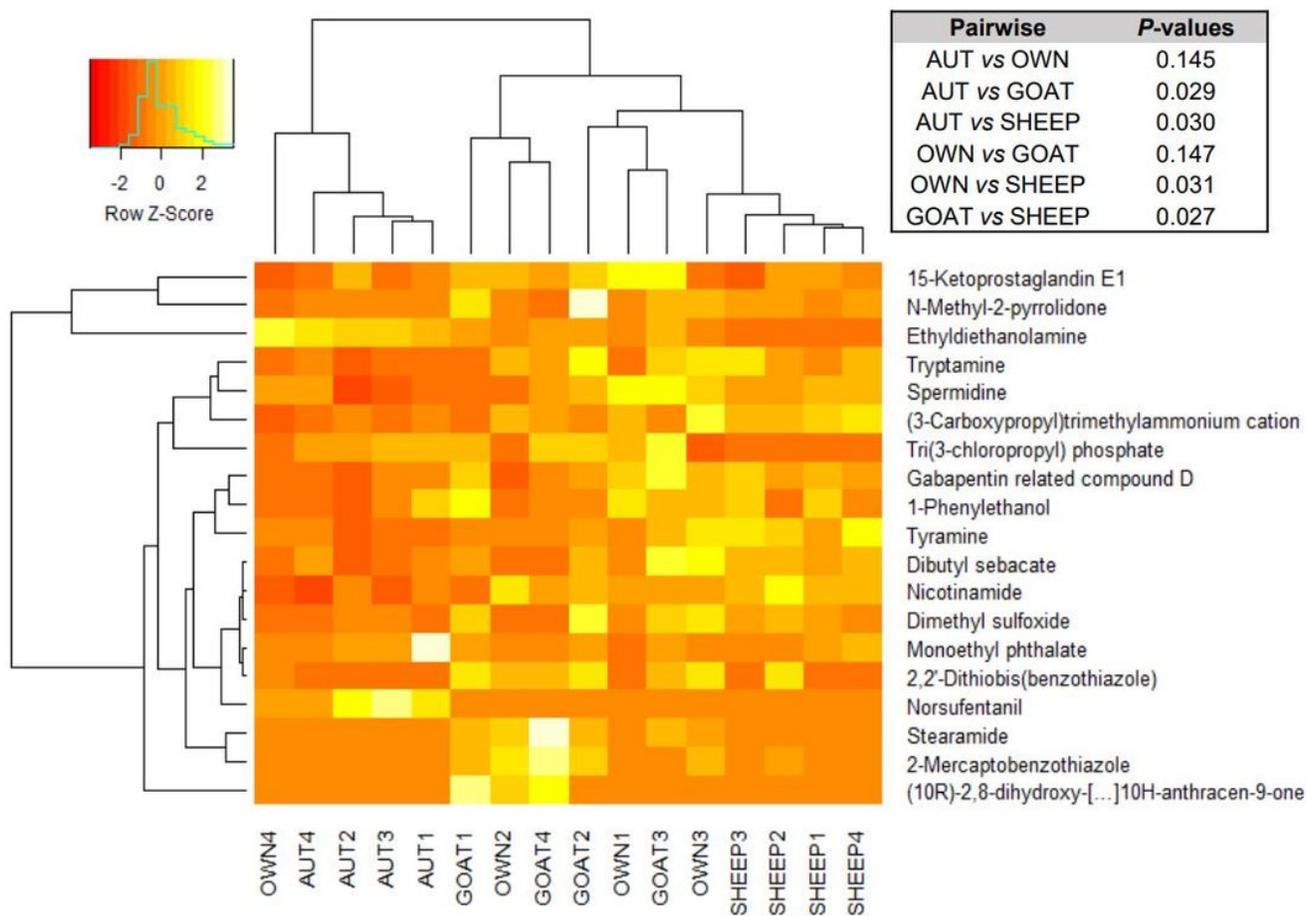
**Figure 4**

Sparse partial least squares discriminant analysis of the prokaryotic communities in rumen content incubated with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT), and sheep saliva (SHEEP).



**Figure 5**

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).



**Figure 6**

Heatmap showing the abundance based on mTIC 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).

## Supplementary Files

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