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Characterization of the ruminal fermentation and microbiome in lambs supplemented with hydrolysable and condensed tannins

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ABSTRACT

This study characterised the response of ruminal fermentation and the rumen microbiome in lambs fed commercial vegetal sources of hydrolysable tannins (HT) and condensed tannins (CT). Forty-four lambs (19.56 ± 2.06 kg) were randomly assigned to either a concentrate diet (CON, n = 8) or CON supplemented with 4% of two HT [chestnut (*Castanea sativa*, HT-c) and tara (*Caesalpinia spinosa*, HT-t)] and CT [mimosa (*Acacia negra*, CT-m) and gambier (*Uncaria gambir*, CT-g)] extracts (all, n = 9) for 75 days pre-slaughter. Tannin supplementation did not influence ruminal fermentation traits. Quantitative PCR demonstrated that tannins did not affect the absolute abundance of ruminal bacteria or fungi. However, CT-m (-12.8%) and CT-g (-11.5%) significantly reduced the abundance of methanogens while HT-t (-20.7%) and CT-g (-20.8%) inhibited protozoal abundance. Ribosomal amplicon sequencing revealed that tannins caused changes in the phylogenetic structure of the bacterial and methanogen communities. Tannins inhibited the fibrolytic bacterium, *Fibrobacter* and tended to suppress the methanogen genus, *Methanosphaera*. Results demonstrated that both HT and CT sources could impact the ruminal microbiome when supplemented at 4% inclusion level. HT-t, CT-m and CT-g extracts displayed specific antimicrobial activity against methanogens and protozoa without compromising ruminal fermentation in a long-term feeding trial.

Keywords: Tannins, ruminal fermentation, microbiome, bacteria, methanogens, metagenomics

INTRODUCTION

Structural composition and functional activity of the rumen microbiome influence the efficiency of ruminal metabolism, which in turn affects ruminant productivity, environmental emissions (methane and nitrogen) and the quality of ruminant-derived foods (McCann, Wickersham and Loor 2014). Several chemical additives and in-feed antibiotics have been developed as rumen modifiers to favourably manipulate ruminal fermentation, methanogenesis, dietary protein degradation and biohydrogenation of dietary fatty acids (Adesogan 2009). Currently, there is a renewed interest in the use of dietary phytochemicals as natural rumen modifiers due to increasing consumer demand for natural food products coupled with global concerns for antibiotic resistance (Patra and Saxena 2009). Tannins, saponins and essential oils are the main classes of phytochemicals that have received attention for their potential to modify ruminal fermentation, methanogenesis, proteolysis and biohydrogenation of fatty acids (Hart, Yanez-Ruiz, Duval, *et al.* 2008; Patra, Min and Saxena 2012).

Tannins are complex polyphenolic compounds that are ubiquitous in several plant species (Makkar 2003) and are broadly divided into hydrolysable tannins (HT) and condensed tannins (CT) based on chemical structure. HT consist of gallic acids esterified to a core polyol and may be further complicated by additional esterification or oxidative crosslinking of the galloyl group whereas CT are flavonoid-based polymers commonly linked by C4-C8 and C4-C6 interflavan bonds (Hagerman 2002). Both HT and CT have diverse and complex structural chemistry. The prevalence of HT and CT varies in different plant sources and a particular type of monomer may also dominate the HT or CT units. Different vegetal sources of tannins can exert beneficial or adverse effects on ruminants depending on factors such as structural complexity, dose-response effect, type of diets, animal species and physiological status (Makkar 2003; Mueller- Harvey 2006). The magnitude of response of ruminal fermentation and feed digestibility can vary between different tannin sources supplemented at similar levels (Jayanegara, Goel, Makkar, *et al.* 2015; Makkar, Blümmel and Becker 1995). Variations in the complex structural features of tannins largely account for the vast discrepancy in the biological potency of different tannin sources on ruminal metabolism and ruminant performance (Patra and Saxena 2011; Waghorn 2008).

The ability of tannins to modify the ruminal microbiome has been associated with decreased ruminal degradation of proteins, reduction of methanogenesis and inhibition of biohydrogenation of fatty acids (Patra, Min and Saxena 2012; Patra and Saxena 2011). Tannins can exhibit bacteriostatic effects, although the interaction of tannins with rumen microbes differs between tannin types given that HT are more susceptible to microbial hydrolysis than CT (Bhat, Singh and Sharma 1998; McSweeney, Palmer, McNeill, *et al.* 2001). Few studies have compared the effect of CT and HT sources on ruminal microorganisms. A tannin-resistant bacterium isolated from the ruminal fluid of goats exhibited a greater tolerance to tannic acid concentration (up to 7%) compared to purified quebracho CT (up to 4%) (Nelson, Pell, Schofield, *et al.* 1995). O'donovan and Brooker (2001) indicated that both tannic acid (HT) and acacia CT inhibited the *in vitro* growth rate of *Streptococcus gallolyticus* (S. caprinus) and *S. bovis* but *Streptococcus gallolyticus* later maintained its growth through the initiation of adaptation mechanisms that differ between tannic acid and acacia CT. The resistance mechanisms of *S. gallolyticus* to tannic acid include induction of gallate decarboxylase activity and secretion of extracellular polysaccharide matrix while the resistance of *S. gallolyticus* to acacia CT involve an unknown mechanism. An *in vitro* ruminal study reported that purified HT (chestnut and sumach) and CT (mimosa and quebracho) decreased the population of methanogens and *Fibrobacter succinogenes* but sumach HT exhibited a greater inhibitory effect on *Ruminococcus flavefaciens* compared to other tannins (Jayanegara, Goel, Makkar, *et al.* 2015). Furthermore, dietary supplementation of chestnut HT and quebracho CT increased the abundance of *Butyrivibrio fibrisolvens* in the rumen of dairy sheep by approximately 3-fold and 5-fold, respectively (Buccioni, Pauselli, Viti, *et al.* 2015). On the other hand, chestnut HT and quebracho CT inhibited the population of *B. proteoclasticus* by 5-fold and 15-fold, respectively (Buccioni, Pauselli, Viti, *et al.* 2015). These studies indicated that there could be a substantial variation in the response of the ruminal microbiome to dietary HT and CT sources.

The *in vivo* effect of different dietary tannins, especially when supplemented at a moderate dosage in a long-term experiment, on the rumen microbial ecosystem is still poorly understood. It was hypothesised that different types of tannins (HT *vs.* CT) would elicit differential impacts on the ruminal fermentation and microbiome. Thus, the present study characterised the ruminal fermentation and microbiome in lambs fed commercial extracts of HT: chestnut (*Castanea sativa*) and tara (*Caesalpinia spinosa*); and CT: mimosa (*Acacia negra*) and gambier (*Uncaria gambir*) for 75 d pre-slaughter. An inclusion rate of 4% tannin extracts was adopted in this experiment because the inclusion of 2.5 to 5% has been suggested as the moderate dosage threshold for the biological potency of tannins that would not impair nutrient intake and animal performance (Mueller- Harvey 2006; Patra and Saxena 2011).

MATERIALS AND METHODS

Animal, diets and experimental design

The feeding trial was carried out at the experimental farm in the University of Catania. The animals were handled according to the European Union legislation for the protection of animals used for scientific purposes (2010/63/EU Directive). Forty-four male cross-bred Sarda x Comisana lambs [initial body weight (BW) 19.56 ± 2.06 kg] were completely randomised to five experimental groups. Each animal was reared

in an individual stall with straw beddings. The animals were adapted to the experimental diets for a period of 9 d by progressive substitution of the weaning feed with the experimental feeds until total replacement of the weaning diet was achieved. Subsequently, the animals were fed for 75 d pre-slaughter. The experimental diets consisted of a commercial concentrate diet (CON, *n* = 8) and four tannin treatments (*n* = 9) containing CON supplemented with 40 g/kg commercial extract sources of two hydrolysable tannins, HT [chestnut (HT-c) and tara (HT-t)] and condensed tannins, CT [mimosa (CT-m) and gambier (CT-g)]. The animals had *ad libitum* access to the concentrate feeds and water.

The concentrate diet consisted of the following ingredients (*as-fed* basis): barley (48.0%), dehydrated alfalfa (15.0%), wheat bran (23.0%), soybean (10.0%), molasses (2%) and mineral-vitamin premix (2%). The chemical composition of the experimental diets is indicated in Table S1 (Supplementary data). Commercial extracts of chestnut (Nutri-P®), tara (Tannino T80®) and mimosa (Mimosa OP®) were obtained from Silvateam (San Michele M.vì, Cuneo, Italy) and gambier (Retan FGC®) was obtained from Figli di Guido Lapi S.pA. (Castelfranco di Sotto, Pisa, Italy). These extracts were selected as the source of each specific type of tannins (HT or CT) based on published information on the chemical composition of their phenolic fractions (Apea-Bah, Hanafi, Dewi, *et al.* 2009; Kardel, Taube, Schulz, *et al.* 2013; Pizzi, Pasch, Rode, *et al.* 2009). Furthermore, these tannins, except CT-g, have been previously evaluated in *in vitro* ruminal fermentation models (Jayanegara, Goel, Makkar, *et al.* 2015; Pellikaan, Stringano, Leenaars, *et al.* 2011). The concentrate diets were supplied in form of pellets and the tannin extracts were incorporated into the diets before pelleting at 40°C. Total phenols and tannins in the diets were analysed as previously described by Gravador, Luciano, Jongberg, *et al.* (2015).

Rumen sampling and determination of fermentation variables

The lambs were slaughtered in a commercial abattoir using captive bolt stunning before exsanguination. The pH of the ruminal digesta was measured immediately after slaughter using a pH-meter (Orion 9106, Orion Research Incorporated, Boston, MA). Two aliquots of approximately 80 g each of ruminal digesta were collected within 20 min of slaughter and immediately placed in dry ice prior to storage at -80°C for further analysis of rumen fermentation and microbial population. One aliquot of the ruminal digesta stored at -80°C was thawed overnight at 4°C and divided into two aliquots for volatile fatty acids (VFA) and ammonia analyses. The thawed aliquots were centrifuged at 1000 g for 20 min at 4°C and 4 ml of the supernatant (2 ml pooled from each of the two aliquots) was added to 1 ml of 25% trichloroacetic acid (TCA) containing 20 mM 2-ethylbutyric acid as an internal standard. The concentration of VFA was determined as previously described (de la Fuente, Belanche, Girwood, *et al.* 2014). Ammonia analysis was performed by diluting the acidified rumen samples with 25% TCA in ratio 4:1, followed by centrifugation at 15,000 g for 15 min and the supernatant was analysed for ammonia concentration. Ammonia concentration was determined in a ChemWell®-T autoanalyser (Awareness Technology Inc., Palm City, FL, USA - Megazyme Cat. No. D-CHEMT) using the method described by Weatherburn (1967).

The protein content and enzymatic activities (carboxymethyl cellulase, xylanase and amylase) were determined in freeze-dried rumen samples (solid and liquid digesta) as described by Belanche, Pinloche, Preskett, *et al.* (2016). Lipase activity was determined by a spectrophotometric assay with *p*-nitrophenyl butyrate (PNPB) as a substrate dissolved in acetonitrile at a concentration of 10mM (Lee, Koh, Kim, *et al.* 1999). Briefly, freeze-dried rumen samples (~ 200 mg) were diluted with potassium phosphate buffer (3.5 mL) and sonicated for 10 min in ice water. The sample extracts were centrifuged at 5000 rpm for 10 min at 4°C. The cell-free supernatant (60 µl) was mixed with PNPB (180 µl) solution and incubated at 39°C for 30 minutes. Lipase activity was measured as the amount of p-nitrophenol (PNP) released by monitoring the change in absorbance on a spectrophotometer (BioTek, Potton, UK) at 405 nm for 30 min. The blank containing sample supernatant (60 µl) and buffer (180 µl) was incubated to correct for non-enzymatic hydrolysis. All enzymatic activities were measured in triplicate. The specific activity of carboxymethyl cellulase, xylanase and amylase were expressed as mg of sugar released/g protein/min while lipase activity was expressed as mM PNP per g of protein per min.

DNA extraction and quantitative PCR analysis

The rumen samples were freeze-dried and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) detergent method (William and Feil 2012) with slight modifications. Lysis of cells was achieved by incubating with sodium dodecyl sulphate (SDS) buffer for 10 min at 95°C and potassium acetate was substituted for phenol in removing proteins. DNA yield was assessed by spectrophotometry (Nanodrop ND-1000 spectrophotometer, Thermo Fisher Scientific, UK). The extracted DNA samples were stored at -80°C prior to further analysis. Quantitative PCR was performed in triplicate to determine the total concentration of bacteria, methanogens, anaerobic fungi and protozoa in the DNA samples using a LightCycler® 480 System (Roche, Mannheim, Germany) as previously described (Belanche, de la Fuente and Newbold 2015). Targeted primers used for qPCR analysis are indicated in Table S2 (Supplementary data).

Ion torrent next-generation sequencing (NGS) and bioinformatics

The extracted DNA samples were amplified at the V1-V2 and V2-V3 hypervariable regions of 16S rRNA for bacterial and methanogen profiling, respectively as detailed by Belanche, Jones, Parveen, *et al.* (2016). For bacterial profiling, amplification of the V1-V2 hypervariable

regions of the 16S rRNA was carried out using bacterial primers (27F and 357R) followed by ion torrent adaptors (Belanche, Jones, Parveen, *et al.* (2016), Table S2 Supplementary data). For methanogens profiling, amplification of the V2-V3 hypervariable region of the 16S rRNA was performed using archaeal primers (86F and 519R) also followed by ion torrent adaptors (Belanche, Jones, Parveen, *et al.* (2016), Table S2 Supplementary data). The resultant PCR amplicons were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter Inc., Fullerton, USA) and DNA concentration was quantified using an Epoch Microplate Spectrophotometer (BioTek, Potton, UK). The amplicon library was further purified using the E-Gel Safe Imager Trans-illuminator with E-Gel Size Select 2% Agarose gels (Life Technologies Ltd., Paisley, UK). The purified DNA libraries were then quantified for DNA yield and detection of artefacts post-PCR amplification using the Agilent 2100 Bioanalyzer with a high sensitivity DNA chip (Agilent Technologies, Ltd., Stockport, UK). The emulsion PCR of the DNA sample libraries was performed using the Ion PGM Template OT2 200 Kit (Life Technologies Ltd, Paisley, UK) following the appropriate manufacturer's guide for users. The bacterial and methanogen amplicon libraries were sequenced on the Ion Torrent PGM (Life Technologies Ltd, Paisley, UK) using the Ion PGM sequencing 316^{TM} Chip v2.

The resultant sequences were multiplexed for identification of barcodes corresponding to each sample and sequences were denoised to remove low-quality datasets and chimeras using MOTHUR software package as previously described (Belanche, Jones, Parveen, *et al.* 2016). The sequences were further clustered into OTUs at 97% identity using the UPARSE pipeline (http://drive5.com/uparse/) (Edgar 2013). The bacterial and methanogen OTU tables were normalised by random subsampling according to the sample with the minimum number of sequences. Taxonomic classification of bacteria and methanogens was carried out by comparison of the 16S rRNA gene sequences against RDP-II (Wang, Garrity, Tiedje, *et al.* 2007) and RIM-DB database (Seedorf, Kittelmann, Henderson, *et al.* 2014). The methanogen sequences were further blasted to RDP-II to remove any OTU classified as bacteria from the OTU table and the taxonomy from the RIM-DB was identified at 80% confidence threshold. Raw sequence reads were deposited at the EBI Short Read Archive (SRA) from the European Nucleotide Archive (ENA) and can be accessed under the study accession numbers: **(EBI accession number will be included here once DNA sequences are deposited).**

Statistical analysis

Data from ruminal fermentation and microbial abundance were analysed as a one-way ANOVA in SPSS (IBM Statistics version 22) using the following linear model:

$$Y_{ij} = \mu + t_i + \varepsilon_{ij}$$

where Y_{ij} = measured response, μ = overall mean effect, t_i = fixed effect of treatment (i = 1, …, 5) and ε_{ij} = experimental error term. Contrast tests were used to assess the overall effect of dietary tannins (control *vs.* HT-c + HT-t + CT-m + CT-g) and the effect of tannin type (HT *vs.* CT: HT-c + HT-t *vs.* CT-m + CT-g). Shapiro-Wilk normality test was applied on qPCR data and the $\log_{10}$ transformation was performed if unequal variances were found. Log-transformed data were subsequently analysed as highlighted for rumen fermentation variables. Significance was considered when $P \leq 0.05$ and a tendency for treatment effect was observed when $0.05 < P \leq 0.10$. When significance was detected, Tukey's HSD test was used for multiple comparisons of treatment means. Pearson's correlation analysis was performed to assess the relationship between the abundance of methanogens and protozoa.

The biodiversity indices of bacterial and methanogen microbiome were calculated using the normalised OTU datasets using PRIMER-v6 ecological software (PRIMER-E Ltd., Plymouth, UK). Permutational multivariate analysis of variance (PERMANOVA) was used to enumerate the structural differences in the bacterial and methanogen communities using Bray-Curtis similarity measurement of log-transformed OTU datasets. The pseudo *F*-statistics and *P*-values were obtained by performing 999 random permutations of residuals under a reduced model using Monte Carlo test as described by Belanche, Jones, Parveen, *et al.* (2016). Non-parametric multi-dimensional scaling plots and dendrogram plot of hierarchical cluster analysis were generated using PRIMER-v6. Heat map and rarefaction curve were constructed using the vegan package in R statistical software (version 3.2.5). Treatment effect on the relative abundances of bacterial and methanogen taxa was analysed as a one-way ANOVA as indicated for qPCR data. Tukey's HSD test was used for multiple comparisons of treatment means when significance was detected and multiple comparisons of relative abundances were adjusted for false positives using Bonferroni correction. The correlations between ruminal fermentation and the bacterial and methanogen population structure were elucidated on ordination plots using canonical correspondence analysis (CCA) generated in R software. *P*-values of the variables were computed using 999 random permutations.

RESULTS

Fermentation parameters and microbial abundance

Dietary treatment tended to influence rumen pH and total VFA concentration (Table 1). Multiple comparisons of treatment groups showed that HT-c elicited significantly lower (P = 0.044, data not shown) rumen pH and higher (P = 0.049, data not shown) total VFA compared to CT-g. Dietary treatment did not affect (P > 0.05) ammonia concentration nor the molar proportion of acetate, propionate, valerate and acetate/propionate ratio, and enzymatic activities. However, CT-m lambs had an increased (P < 0.05) molar proportion of butyrate compared to CON and CT-g animals. HT-c lambs had a lower molar proportion of *iso*-butyrate compared to CT-m lambs and lower *iso*-valerate than lambs fed the CT-m or CT-g. No significant effect of tannin supplementation on ruminal fermentation traits except for a tendency (P = 0.066) for tannin treatments to increase the molar proportion of butyrate. HT extracts lowered ruminal pH and increased the concentration of total VFA (+31.6%) compared to CT extracts. Moreover, HT extracts tended (P = 0.074) to elevate molar proportion of propionate but decreased (P < 0.05) the molar proportion of *iso*-butyrate and *iso*-valerate compared to CT extracts. However, enzymatic assays indicated that there was no effect of dietary tannins on specific activities of carboxymethyl cellulase, xylanase, amylase or lipase.

Real-time PCR indicated that absolute abundance of bacteria and fungi were comparable between treatments but tannin treatments reduced the abundance of methanogens (P = 0.002) and protozoa (P = 0.003) (Table 2). In comparison to CON, the abundance of methanogens was significantly inhibited by CT-m (-12.8%) and CT-g (-11.5%) while the abundance of protozoa was reduced by HT-t (-20.7%) and CT-g (-20.8%). However, the inhibitory effect of tannins on methanogen and protozoa abundance was similar (P > 0.05) between HT and CT extracts.

(Table 1 and 2 here)

Bacterial microbiome

Ion torrent NGS of bacterial 16s rDNA genes yielded approximately 1.6 million raw sequences clustered into 2027 OTUs across 44 samples. Subsequently, the NGS data sets were normalised resulting into 936,176 high-quality sequences clustered into 2010 unique OTUs, with an average of 21,276 sequences per sample. The rarefaction curves plateaued, suggesting that sampling of the rumen environments have comparable sequencing depth across treatments (Figure S1, Supplementary data). Biodiversity indices of the rumen bacterial community were unaffected by dietary treatment (Table 2). Non-metric multi-dimensional scaling (NMDS) plot indicated a slight clustering separating HT-c and other dietary treatments (Figure 1a). PERMANOVA showed that dietary treatment altered the bacterial community structure and tannin supplementation tended to influence the bacterial community structure (Table 3). Pairwise comparisons revealed that bacterial community structure differs between CON and HT-c as well as between CT-m and CT-g. Moreover, there were tendencies for bacterial community differences between CON and CT-m and between HT-c and CT-g (Table 3).

Taxonomic classification indicated that *Bacterioidetes* (40.7%) was the predominant phylum followed by *Proteobacteria* (38.5%), *Firmicutes* (15.2%), *Fibrobacteres* (2.3%), *Spirochaetes* (1.2%), *Actinobacteria* (0.4%), *SR1* (0.3%) and *Tenericutes* (0.2%), with a limited number of unclassified sequences (0.9%) (Table 4). At the genus level, *Prevotella* (28.6%) and *Ruminobacter* (21.9%) were the predominant groups and heat map illustrated no distinct grouping of the genera across treatments (Figure S2, Supplementary data). The effect of dietary treatment on phylogenetic composition was only obvious on the relative abundance of *Bacterioidetes* and *Fibrobacteres* phyla. The abundance of *Bacterioidetes* was lower in CT-g compared to CT-m and this was mediated by a shift from genus *Prevotella* (*Bacterioidetes*) to *Ruminobacter* (*Proteobacteria*) (Table 4). Dietary tannins significantly decreased *Fibrobacteres*, *Fibrobacteraceae* and *Fibrobacter* at the phylum, family and genus levels, respectively (Table 4). The inhibitory effect of tannins was more apparent in HT-c when compared to CON (Table 4). The ordination plot of the canonical correspondence analysis (CCA) illustrated the correlation between the rumen bacterial community and the fermentation and microbial parameters (Figure 2a). There was a clustering of samples from HT-c-fed animals. The CCA plot indicated that positive correlations of butyrate (P = 0.001), acetate (P = 0.058), protein content (P = 0.002), acetate/propionate ratio (P = 0.001) and bacterial diversity (P = 0.001) appeared to be associated with the bacterial community in HT-c samples (Figure 2a and Table S3, Supplementary data). However, the correlation of amylase activity tended (P = 0.075) to be associated with the rumen bacterial community in lambs fed CON, HT-t, CT-m and CT-g.

(Table 3 and 4 here)
(Figure 1 and 2 here)

Methanogen microbiome

Sequencing of methanogenic 16s rDNA genes produced about 3 million sequences clustered into 57 OTUs across 44 samples after quality filtering. The NGS data sets were normalised resulting into 572,792 high-quality sequences clustered into 57 unique OTUs, with an average of 13,018 sequences per sample. Methanogen diversity indices were unaffected by dietary treatments (Table 2). However, Simpson's index suggests that dietary tannins tended to promote higher diversity in methanogen community. Visualisation of the methanogen community data sets on NMDS plot showed that there was no clear clustering between diets (Figure 1b). Dietary treatment elicited variable effects on the methanogen community structure but neither tannins nor tannin types (HT *vs.* CT) had a significant impact (Table 3). Pairwise comparison of the methanogen community structure revealed that HT-c differed from HT-t and CT-g diets whereas CT-m differed from HT-t and CT-g diets. Additionally, there was a tendency for differences when CON was compared to CT-m methanogen community structure.

Euryarchaeota was the only identified phylum, accounting for 99.1% of methanogen abundance. The predominant families were *Methanobacteriaceae* (30.8%, *Methanobacteria*) and *Methanomassiliicoccaceae* (5.9%, class *Thermosplasmata*) while the abundances of *Methanocorpusculaceae* and *Methanosarcinaceae* (class *Methanomicrobia*) were less than 0.1% (Table 5). *Methanobrevibacter* (29.0%) dominate the genus level followed by *Methanomassiliicoccus* (5.9%) and *Methanosphaera* (1.8%). Neither diets, tannins nor tannin type influence ($P > 0.05$) the relative abundance of methanogen taxa (Table 5). However, dietary tannins tended ($P = 0.080$) to inhibit genus *Methanosphaera*. Methanogen microbiome significantly correlated with amylase activity ($P = 0.047$) and tended to correlate with bacteria abundance ($P = 0.062$) without apparent association to any dietary treatment (Figure 2b and Table S4, supplementary data).

(Table 5 here)

DISCUSSION

Dietary tannins may have potential as natural rumen modifiers but their effect on ruminal metabolism varies with different tannin sources (Patra, Min and Saxena 2012; Patra and Saxena 2011). HT are more susceptible to microbial hydrolysis than CT (Bhat, Singh and Sharma 1998) and the products of tannin degradation may influence ruminal metabolism (McSweeney, Palmer, McNeill, *et al.* 2001). The current study, therefore, hypothesised that different types of tannins (HT *vs.* CT) would elicit differential impacts on the ruminal fermentation and microbiome.

Ruminal fermentation

Tannins can react with proteins to form complexes that are resistant to ruminal degradation and/or inhibit the growth and activity of proteolytic bacteria, thereby increasing the post-ruminal flow of non-ammonia N and improving N utilisation efficiency in ruminants (Makkar 2003; Waghorn 2008). In the current study, none of the dietary tannins affected NH_3-N concentration in contrast with several *in vivo* studies supporting the effect of tannins in reducing protein degradation in the rumen (Al- Dobaib 2009; Broderick, Grabber, Muck, *et al.* 2017; Puchala, Min, Goetsch, *et al.* 2005). The formation of stable and insoluble tannin-protein complexes is induced at pH 3.5 – 7.0 (Jones and Mangan 1977), which is consistent with the rumen pH of lambs measured in this study. Similarly, the tannin/protein (w/w) intake ratios for lambs fed HT-c (1:7.1), HT-t (1:5.8) and CT-m (1:6.4) are within the range (≥1:10-12, w/w) of tannin concentration required to decrease ruminal proteolysis (Jones and Mangan 1977; Tanner, Moore and Larkin 1994). However, the effect of tannins in inhibiting ruminal proteolysis is inconsistent. The inclusion of 20 – 40 g/kg DM extracts of quebracho-CT (Piñeiro- Vázquez, Canul- Solis, Alayón- Gamboa, *et al.* 2017) or chestnut-HT and valonea-HT (Wischer, Greiling, Boguhn, *et al.* 2014) did not affect ruminal NH_3-N nor protein utilisation efficiency in cattle and sheep, respectively. The protein precipitating capacity of tannins may be affected by their structural features (Kraus, Yu, Preston, *et al.* 2003), and the degree of tannin affinity to proteins can be influenced by molecular weight, isoelectric point, compatibility of binding sites and protein tertiary structure (Patra and Saxena 2011; Reed 1995). Thus, the interaction between tannin source and the type of feed protein could influence protein precipitation in the rumen (Giner- Chavez, Van Soest, Robertson, *et al.* 1997; Zeller, Sullivan, Mueller-Harvey, *et al.* 2015). Lorenz, Alkhafadji, Stringano, *et al.* (2014) indicated that protein type influences the concentration of tannins that could induce precipitation, suggesting that higher inclusion level of the tannin extracts may be required to decrease rumen protein degradation in our study. Furthermore, the comparison of tannin effect across *in vivo* studies may be complicated by differences in the digestion kinetics of the basal diet (concentrate *vs.* forage-based diet) and ruminant species. Concentrate diets are rapidly digested in the rumen with a shorter transit time compared to forage-based diets and ruminal digestion kinetics also varies among ruminant species (Colucci, Macleod, McMillan, *et al.* 1984; Huhtanen, Ahvenjärvi, Weisbjerg, *et al.* 2006). These variations result in a temporal factor limiting the formation of tannin-protein complex in the rumen especially when tannins are included in concentrate diets. For instance, HT-c did not affect ruminal proteolysis when supplemented in a concentrate diet of lambs and sheep in the present study and that of Wischer, Greiling, Boguhn, *et al.* (2014), respectively. In contrast, HT-c reduced ruminal protein degradation when included in a forage-based diet of cattle (Tabacco, Borreani, Crovetto, *et al.* 2006).

Typically, ruminants derive approximately 70% of their energy requirement from VFA (mainly acetate, propionate and butyrate) produced from microbial fermentation of feeds in the rumen (Bergman 1990). Tannin concentration is often considered the most influential factor affecting ruminal fermentation (Patra and Saxena 2011). However, it is evident that the response could also vary between different tannin sources supplemented at a similar dosage (Getachew, Pittroff, Putnam, *et al.* 2008; Makkar, Blümmel and Becker 1995). High concentration of tannins can inhibit feed digestibility and ruminal fermentation by forming complexes with lignocellulose or by directly inhibiting rumen micro-organisms and microbial enzymes (Bae, McAllister, Yanke, *et al.* 1993; Chung, Lu and Chou 1998; Nsahlai, Fon and Basha 2011). In the present study, 4% inclusion of the HT and CT extracts modified the rumen microbiota without compromising overall ruminal fermentation. Indeed, greater total VFA concentration was measured in HT-fed lambs compared to lambs fed CT sources or the control diet. Min, Wright, Ho, *et al.* (2014) also reported that dietary addition of 100 g/d chestnut-HT resulted in a higher total VFA than quebracho-CT and the control diet in grazing goats. However, Jayanegara, Goel, Makkar, *et al.* (2015) showed that increasing dosage (0.5, 0.75 and 1.0 mg/ml) of either purified HT (chestnut and sumach) or CT (mimosa and quebracho) resulted in a quadratic decrease of *in vitro* dry matter digestibility, with a tendency for a more pronounced decrease in CT sources. Nonetheless, the increase in total VFA concentration elicited by HT extracts in this study may be related to their possible hydrolysis into derivates that are metabolised into fermentation products. Chestnut and tara tannins have been shown to be susceptible to biodegradation induced by bacterial tannase activity (Deschamps and Lebeault 1984; Deschamps, Otuk and Lebeault 1983). The biodegradation of HT and its monomers, such as gallic acid, involves decarboxylation into pyrogallol that is further transformed to several intermediates in step-wise enzymatic reactions, to form acetate and butyrate (Bhat, Singh and Sharma 1998). However, the metabolism of polyphenols could limit their bioavailability and bioactivity for modulating gut microbiota (Ozdal, Sela, Xiao, *et al.* 2016). This may probably explain the limited effect of HT-c in terms of response of the rumen microbiota. Further investigation is required to elucidate the biochemical mechanism involved in the interaction between rumen microbes and HT sources, and the consequent effect on ruminal fermentation.

Molecular quantification of rumen microbial populations demonstrated that tannins did not influence the abundance of bacteria and fungi but inhibited methanogen and protozoal numbers. Tannins could exert anti-microbial effect through multiple mechanisms including induction of membrane disruption; eliciting morphological changes in cell wall via interaction with extracellular enzymes and cell wall; decreasing availability of cations to microbes via chelation; direct effect on the metabolism of microbes and causing deficit of substrates for microbial proliferation (Patra and Saxena 2011; Scalbert 1991). Methanogenic archaea are responsible for producing methane as a by-product of ruminal fermentation and rumen methane is a potent greenhouse gas that contributes significantly to global warming (Morgavi, Forano, Martin, *et al.* 2010). Moreover, methane losses constitute 2 to 12% loss of gross energy intake in ruminants and could undermine animal productivity (Johnson and Johnson 1995). Methanogens may exist either as free-living or associated with protozoa in a symbiotic relationship (Janssen and Kirs 2008). The roles of protozoa in methanogenesis include the supply of H_2 as a substrate for CH_4 formation; serving as hosts for methanogens and protecting methanogens from oxygen toxicity (Newbold, de la Fuente, Belanche, *et al.* 2015). Indeed, there is evidence that suggests a positive relationship between ruminal methanogenesis and the abundance of methanogens and protozoa (Belanche, de la Fuente and Newbold 2015; Wallace, Rooke, Duthie, *et al.* 2014). In agreement with the present study, Pearson correlation analysis indicated that there was a positive correlation ($r = 0.423$; $P < 0.01$) between the abundance of methanogens and protozoa. Thus, strategies that could reduce the abundance of methanogens and protozoa could be useful to mitigate enteric methane emission in ruminants (Morgavi et al. 2010). The anti-methanogenic potential of tannins largely depend on sources and concentration (Goel and Makkar 2012; Jayanegara, Leiber and Kreuzer 2012) but there is a limited information on their long-term persistence effect. The anti-methanogenic effect of different tannins has been attributed to their inhibitory effect on methanogen and protozoa population (Bhatta, Uyeno, Tajima, *et al.* 2009; Tan, Sieo, Abdullah, *et al.* 2011). Given that the tannins were fed for 75 d in the present study, the antimicrobial effect of HT-t, CT-m and CT-g highlight these tannins as potential anti-methanogenic agents that could exhibit long-term persistence. Interestingly, CT-g had the most consistent effect on both methanogen and protozoa abundances but there is no available study assessing its anti-methanogenic effect in ruminants. The inability of HT-c to inhibit methanogen and protozoa abundance may explain the transient anti-methanogenic effect of chestnut extract when supplemented (1.7 g/kg BW0.75) in the diet of sheep for 85 d (Wischer, Greiling, Boguhn, *et al.* 2014). The authors postulated that the rumen microbes could have adapted to HT extracts from chestnut and valonea after 2 weeks of supplementation in two separate experiments conducted over a duration of 85 d and 190 d (Wischer, Greiling, Boguhn, *et al.* 2014). However, this argument contradicts the observation that dietary addition of 30 g/kg of chestnut extract fed over a duration of 60 d decreased methane emission with a concomitant reduction of methanogen and protozoa populations in sheep (Liu, Vaddella and Zhou 2011).

Goel and Makkar (2012) suggested that HT sources decreased methane emissions through a direct inhibitory effect on growth and/or activity of methanogens- and/or hydrogen-producing microbes while CT exhibit an indirect effect by reducing hydrogen availability via depression of fibre digestion. Our results suggest that a wide range of tannin sources could inhibit methanogens and protozoa regardless of their chemical type. Methane emission was not quantified in the present study, however, our results highlight the need for future studies to particularly evaluate the *in vivo* anti-methanogenic activity of CT-g. Moreover, it would be useful to investigate the combination of these tannins given that vegetal sources

containing both HT and CT could exhibit more potent anti-methanogenic activity than sources containing a single tannin type (Bhatta, Uyeno, Tajima, *et al.* 2009). A possible reason could be related to the synergistic effect of different tannins to inhibit either methanogen or protozoa abundance.

Bacterial microbiome

As confirmed by our sequence-based data, *Bacteroidetes*, *Proteobacteria* and *Firmicutes* are the main bacteria phyla in the rumen even though their proportion can vary with different diets (McCann, Wickersham and Loor 2014). Tannins can modify bacterial structure and phylogeny without affecting the abundance of ruminal bacteria (Saminathan, Sieo, Gan, *et al.* 2016b). In this study, supplemental tannins altered the phylogenetic structure and composition of the bacteria community without impairing ruminal fermentation. HT-c was the only tannin that had a large effect on the bacterial community when compared to CON treatment. Moreover, the CCA ordination plot (Figure 2a) indicated a subtle difference between HT-c and other dietary treatments, with respect to the interplay between the rumen bacterial community and fermentation variables.

The disparity in the bacterial community structure between CT-g and CT-m was associated with a shift from *Prevotella* and *Paraprevotella* to *Ruminobacter*. Despite the bacterial shifts, CT-g and CT-m displayed similar ruminal fermentation traits which could be attributed to the fermentative redundancy between microbial groups (Weimer 2015). Indeed, the rumen ecosystem can maintain similar metabolic function despite differences in microbial communities (Taxis, Wolff, Gregg, *et al.* 2015). The decrease in the abundance of *Fibrobacter* in tannin-supplemented lambs further confirmed the profound inhibitory effect of dietary tannins on fibrolytic bacteria and fibre digestibility (Bae, McAllister, Yanke, *et al.* 1993; Jayanegara, Goel, Makkar, *et al.* 2015; Sivakumaran, Molan, Meagher, *et al.* 2004). This suggests that the current inclusion level of tannin extracts may impair ruminal digestion of high-fibre diets in contrast to the concentrate diet fed in the present study. Tannins may have a selective effect on fibrolytic bacteria depending on dosage and type of tannins (Patra, Min and Saxena 2012). In the present study, HT-c specifically inhibited *Fibrobacter* in comparison to CON. However, a similar commercial chestnut extract did not affect the abundance of *Fibrobacter succinogenes* when 30 g/kg was supplemented in sheep diets for 60 d (Liu, Vaddella and Zhou 2011). The longer duration of supplementation (75 d *vs.* 60 d) and greater dosage (40 g/kg *vs.* 30 g/kg) could account for the discrepancy in the effect of HT-c on *Fibrobacter* between the present study and that of Liu, Vaddella and Zhou (2011). Additionally, the structural features of tannins could play a dominant role in the effect of different tannins on fibrolytic bacteria. Saminathan, Sieo, Gan, *et al.* (2016b) suggested that the abundance of *Fibrobacter* increased with increasing molecular weight (469.6 – 1265.8 Da) of CT fractions from *Leucaena leucocephala* hybrid.

Methanogen microbiome

The authors believe that this is the first study to characterise the *in vivo* effect of HT-c, HT-t, CT-m and CT-g on the rumen methanogen communities. It has been shown that potential anti-methanogenic effect of tannins could be related to a decrease in the abundance of methanogens and/or alteration in methanogen population structure (Longo, Abdalla, Liebich, *et al.* 2013; Tan, Sieo, Abdullah, *et al.* 2011). However, the present study indicated that dietary tannins did not exert a significant effect on methanogen diversity or community structure in comparison to CON. Saminathan, Sieo, Gan, *et al.* (2016a) recently utilised different CT fractions from *Leucaena leucocephala* hybrid to demonstrate that the molecular weights (469.6 – 1265.8 Da) of tannins could play a role in their modulatory effect on *in vitro* methanogen community structure. Accordingly, CT fractions with higher molecular weights resulted in a greater impact on methane production and methanogen community structure (Saminathan, Sieo, Abdullah, *et al.* 2015; Saminathan, Sieo, Gan, *et al.* 2016a). This highlights the need for future studies to explore the diversity in structural chemistry of tannins with respect to their bioactivity to modulate rumen microbiota.

As confirmed in the present study, *Methanobrevibacter* may be the most dominant methanogen genus in the rumen (Janssen and Kirs 2008) but tannins did not affect the relative abundance of this methanogen phylotype. Notably, tannins exhibited a specific tendency to reduce the abundance of *Methanosphaera* by approximately 40% while HT-c supplementation, in particular, resulted in a decrease of approximately 61% compared to CON. *Methanosphaera* spp. are important methanogens that can utilise hydrogen and methyl groups as precursors for methane formation in the rumen (Tapio, Snelling, Strozzi, *et al.* 2017). Min, Solaiman, Shange, *et al.* (2014) reported that dietary inclusion of pine bark (3.2% CT diet DM) reduced the relative abundance of *Methanosphaera* by 37% in faecal samples from goats. It is possible that tannins elicit apparent effects on methanogens at the species level particularly on the predominant genera, *Methanobrevibacter* and *Methanosphaera* (Li, Liu, Jin, *et al.* 2013). Nonetheless, this is currently constrained by the limited genomic database on methanogen diversity. Overall, our data suggest that the potential anti-methanogenic effect of these supplemental tannins could be more related to their inhibitory effect on methanogen abundance rather than modulation of methanogen population structure.

In conclusion, this study demonstrates that HT-t, CT-m and CT-g extracts displayed specific antimicrobial activity against methanogens and protozoa without compromising ruminal fermentation and animal productivity in a long-term feeding trial. Further *in vivo* studies are necessary to examine the effect of these tannins, particularly CT-g, in relation to their long-term persistence in reducing enteric methane emission in ruminants. Ruminal degradation of HT sources, such as HT-c, should be further investigated as a factor influencing their modulatory effect on

rumen microbiota and fermentation. Moreover, both HT and CT extracts could impact the ruminal microbiome when supplemented at moderate levels but their negative effect on fibrolytic bacteria should be considered when fed with high-fibre diets.

SUPPLEMENTARY DATA

Supplementary data file is available online at FEMS-MC.

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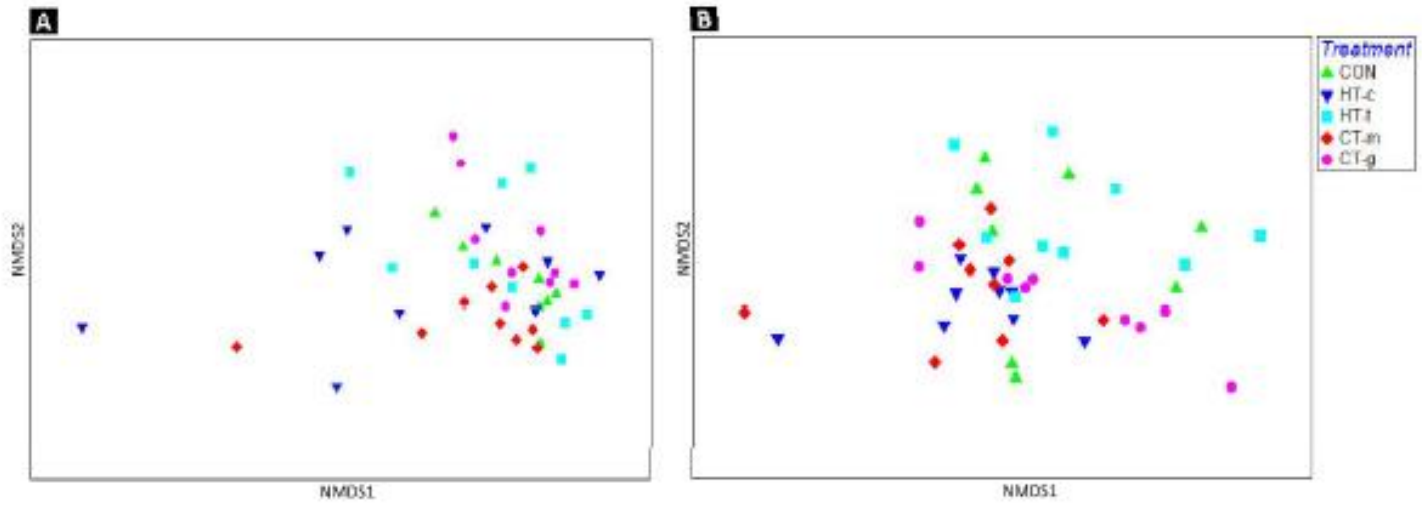

Figure 1. (A) Non-parametric multi-dimensional scaling (NMDS) plot (stress = 0.14) of rumen bacterial communities in lambs fed different tannin extracts; **(B)** NMDS plot (stress = 0.15) of rumen methanogen communities in lambs fed different tannin extracts. Dietary treatments are: CON, control; HT-c, chestnut hydrolysable tannin; HT-t, tara hydrolysable tannin; CT-m, mimosa condensed tannin; CT-g, gambier condensed tannin. NMDS plot was produced from a resemblance matrix created using Bray-Curtis similarity of log-transformed OTU data sets.

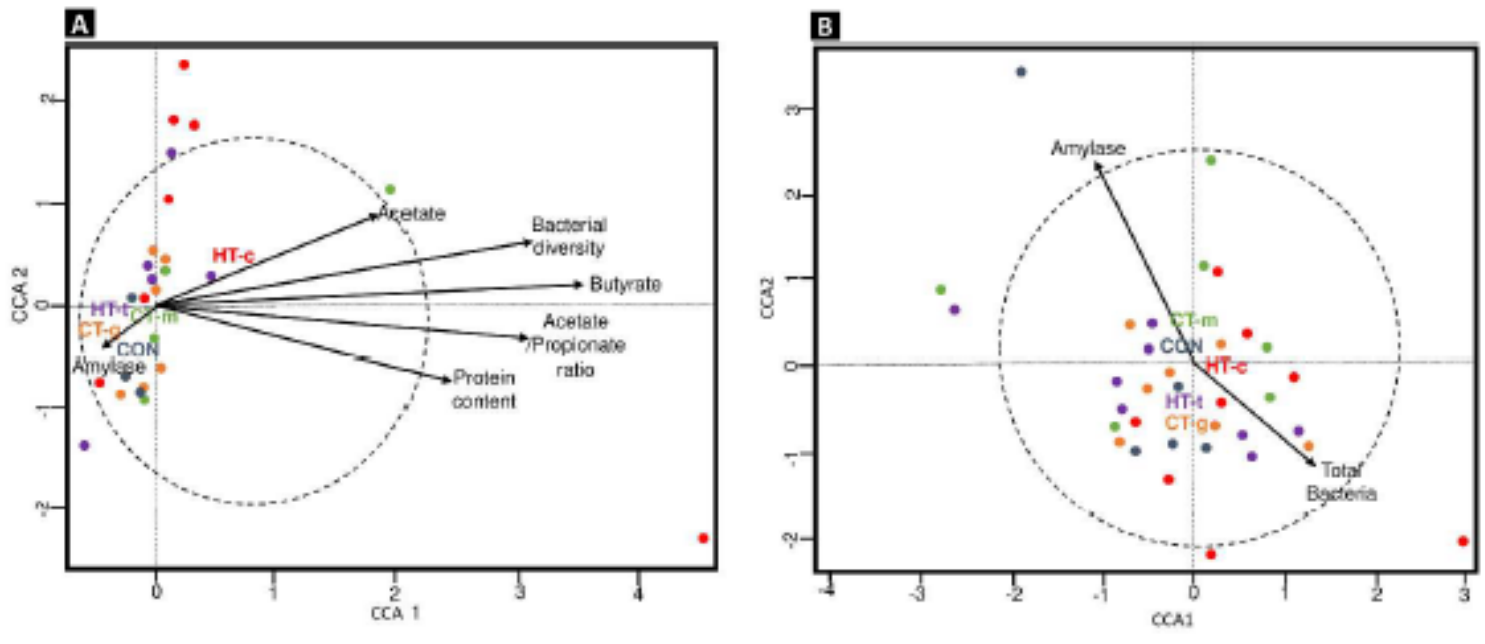

Figure 2. Canonical correspondence analysis (CCA) describing the correlations between: **(A)** rumen bacterial community structure and fermentation and microbial variables; **(B)** rumen methanogen community structure and fermentation and microbial variables. The arrows indicate the gradient direction and their length is relative to the proportion of correlation. Arrows longer than the dotted circle signify correlation significance ($P < 0.05$). Coloured dots represent distribution pattern of the animals fed each dietary treatments: CON, control; HT-c, chestnut hydrolysable tannin; HT-t, tara hydrolysable tannin; CT-m, mimosa condensed tannin; CT-g, gambier condensed tannin.

Table 1. Effect of different tannin extracts on ruminal pH and fermentation characteristics in lambs

Parameter	Dietary treatment[1]							Contrast[2] (*P*-value)	
	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
Rumen pH	6.50	6.01	6.41	6.54	6.63	0.073	0.052	0.572	0.017
NH_3-N (mMol/L)	13.67	13.24	15.98	15.54	15.78	0.899	0.818	0.546	0.611
Total VFA (mMol/L)	55.15	77.45	55.30	46.74	44.09	3.982	0.055	0.939	0.015
Molar proportion of VFA									
Acetate	50.67	53.87	51.04	51.08	52.92	0.678	0.506	0.383	0.766
Propionate	32.73	30.85	32.29	26.12	29.53	0.949	0.179	0.212	0.074
Iso-butyrate	3.68^{ab}	2.09^{b}	3.33^{ab}	3.43^{ab}	4.18^{a}	0.221	0.031	0.430	0.019
Butyrate	6.52^{b}	9.24^{ab}	7.77^{ab}	11.80^{a}	6.56^{b}	0.543	0.005	0.066	0.523
Iso-valerate	2.65^{ab}	1.29^{b}	2.41^{ab}	3.74^{a}	3.42^{a}	0.241	0.006	0.905	0.001
Valerate	3.76	2.67	3.16	3.82	3.39	0.210	0.414	0.368	0.144
Acetate/propionate ratio	1.59	2.08	1.59	2.11	1.88	0.118	0.475	0.302	0.543
[3]**Specific enzymatic activity**									
Carboxymethyl cellulase	34.92	45.10	42.86	43.61	47.13	2.780	0.733	0.194	0.826
Xylanase	129.15	130.71	166.90	128.09	161.26	13.315	0.812	0.623	0.892
Amylase	77.80	51.52	69.29	47.14	77.50	7.478	0.577	0.407	0.909
Lipase	2.46	2.69	2.72	2.41	4.04	0.291	0.376	0.505	0.425

VFA: Volatile fatty acids; SEM: Standard error of mean

[1]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.

[2]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).

[3]Specific activity of carboxymethyl cellulase, xylanase and amylase were expressed as mg of sugar released per g of protein per min while lipase activity was expressed as mM PNP per g of protein per min.

Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.

Table 2. Effect of different tannin extracts on microbial numbers, and bacterial and methanogen biodiversity indices in the rumen of lambs

Parameter	Dietary treatments[2]							Contrast[3] (*P*-value)	
	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
Microbial numbers[1]									
Bacteria (log copy/mgDM)	8.83	8.67	8.90	8.69	8.86	0.040	0.251	0.593	0.906
Methanogens (log copy/mgDM)	6.63^{a}	5.97^{ab}	6.13^{ab}	5.78^{b}	5.87^{b}	0.088	0.020	0.002	0.211
Fungi (log copy/mgDM)	3.19	3.42	2.37	2.27	2.90	0.233	0.456	0.458	0.557
Protozoa (log copy/mgDM)	8.37^{a}	6.86^{ab}	6.64^{b}	7.11^{ab}	6.63^{b}	0.203	0.039	0.003	0.771
Bacterial diversity indices									
Number of OTU	441.25	516.44	464.33	552.22	472.56	16.093	0.195	0.147	0.529
Pielou's evenness index	0.58	0.58	0.55	0.58	0.54	0.009	0.299	0.374	0.803
Shannon's index	3.55	3.63	3.36	3.67	3.32	0.063	0.272	0.749	0.997
Simpson's index	0.91	0.91	0.89	0.91	0.88	0.006	0.154	0.267	0.747

Methanogen diversity indices									
Number of OTU	32.38	34.00	33.56	34.11	32.22	0.478	0.610	0.389	0.572
Pielou's evenness index	0.37	0.43	0.38	0.42	0.38	0.012	0.344	0.267	0.929
Shannon's index	1.29	1.53	1.33	1.49	1.34	0.042	0.279	0.229	0.875
Simpson's index	0.57	0.72	0.60	0.68	0.63	0.019	0.081	0.070	0.812

OTU: Operational taxonomic unit; SEM: Standard error of mean

[1]Data were log-transformed to achieve normality; DM, dry matter of freeze-dried rumen content

[2]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.

[3]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).

Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.

Table 3. Effect of different tannin extracts on rumen bacterial and methanogen community structure in lambs

	Similarity	Pseudo-F	*P*-value
Bacterial community[1]			
Treatment		1.60	0.005
Contrasts			
Tannin[2]		1.47	0.096
Tannin type[3]		1.28	0.127
Pair wise comparison of dietary treatment[4]			
CON *vs.* HT-c	37.92	1.49	0.038
CON *vs.* HT-t	44.45	1.08	0.330
CON *vs.* CT-m	44.87	1.37	0.051
CON *vs.* CT-g	46.71	1.21	0.133
HT-c *vs.* HT-t	36.60	1.16	0.195
HT-c *vs.* CT-m	38.11	1.24	0.142
HT-c *vs.* CT-g	38.28	1.34	0.073
HT-t *vs.* CT-m	41.48	1.20	0.156
HT-t *vs.* CT-g	43.16	1.06	0.327
CT-m *vs.* CT-g	42.89	1.44	0.039
Methanogen community[1]			
Treatment		1.91	0.005
Contrasts			
Tannin[2]		1.01	0.392
Tannin type[3]		1.02	0.411
Pair wise comparison of dietary treatment[4]			
CON *vs.* HT-c	73.26	1.26	0.131
CON *vs.* HT-t	72.42	0.85	0.634
CON *vs.* CT-m	71.52	1.34	0.093
CON *vs.* CT-g	70.90	1.28	0.134
HT-c *vs.* HT-t	71.84	1.65	0.025
HT-c *vs.* CT-m	75.64	0.92	0.535
HT-c *vs.* CT-g	71.70	1.71	0.019

HT-t *vs.* CT-m	71.16	1.50	0.035
HT-t *vs.* CT-g	70.90	1.31	0.122
CT-m *vs.* CT-g	70.42	1.68	0.023

[1]Higher Pseudo-F and lower similarities and *P*-values imply greater differences in the rumen bacterial community structure

[2]Tannin (contrast of overall effect of tannin: CON *vs.* HT-c + HT-t + CT-m + CT-g);

[3]Tannin type (contrast of effect of HT *vs.* CT: HT-c + HT-t *vs.* CT-m + CT-g);

[4]Dietary treatments: CON: control; HT-c: chestnut hydrolysable tannin; HT-t: tara hydrolysable tannin; CT-m: mimosa condensed tannin; CT-g: hambier condensed tannin.

Table 4. Effect of different tannin extracts on relative abundance (%) of bacteria taxa ≥ 0.2% of average abundance in the rumen of lambs

			Dietary treatment[1]							Contrast[2] (*P*-value)	
Phyla	Family	Genus	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
Bacteroidetes			40.10[ab]	39.12[ab]	40.53[ab]	49.04[a]	34.72[b]	1.470	0.028	0.830	0.495
	Porphyromonadaceae		3.14	3.53	3.21	2.71	4.55	0.369	0.602	0.713	0.755
		Barnesiella	2.75	1.46	2.50	1.65	3.82	0.364	0.242	0.673	0.348
	Prevotellaceae		31.38[ab]	28.11[b]	32.93[ab]	39.81[a]	23.71[b]	1.436	0.003	0.939	0.652
		Prevotella	28.30[ab]	25.50[b]	30.27[ab]	37.04[a]	22.08[b]	1.345	0.003	0.889	0.516
		Paraprevotella	0.51	0.55	0.30	0.40	0.22	0.055	0.306	0.328	0.360
		Hallella	0.45	0.35	0.28	0.60	0.22	0.056	0.219	0.550	0.449
		Solobacterium	0.55	0.36	0.25	0.39	0.43	0.079	0.849	0.367	0.573
	Rikenellaceae		1.99	0.12	0.72	0.04	2.94	0.527	0.331	0.448	0.359
		Rikenella	1.99	0.12	0.72	0.04	2.94	0.527	0.331	0.448	0.359
	Marinilabiliaceae		0.13	0.20	0.08	0.30	0.42	0.081	0.714	0.590	0.248
Proteobacteria			37.72	39.56	40.33	28.23	46.67	2.166	0.095	0.855	0.586
	Succinivibrionaceae		34.51	33.94	37.07	26.33	43.12	2.076	0.133	0.907	0.860
		Ruminobacter	20.84[ab]	21.05[ab]	23.27[ab]	13.77[b]	30.63[a]	1.784	0.043	0.756	0.991
		Succinivibrio	0.16	2.26	0.40	3.65	0.19	0.795	0.559	0.488	0.742
		Anaerobiospirillum	6.20	3.37	5.14	4.47	2.60	1.106	0.875	0.442	0.775
Firmicutes			14.47	15.61	14.13	16.42	15.12	0.677	0.847	0.640	0.564
	Ruminococcaceae		4.11	5.35	4.55	5.01	5.11	0.286	0.704	0.247	0.869
		Ruminococcus	1.40	0.65	1.27	0.81	1.42	0.138	0.259	0.304	0.629
		Acetivibrio	0.27	0.42	0.82	0.51	0.82	0.113	0.464	0.211	0.867
	Lachnospiraceae		2.06	2.63	2.80	2.71	2.72	0.135	0.472	0.070	0.996
		Lachnospiracea incertae sedis	0.22	0.31	0.54	0.48	0.49	0.059	0.391	0.128	0.633
		Roseburia	0.89	0.83	1.06	0.47	0.84	0.094	0.387	0.726	0.176
		Butyrivibrio	0.39[ab]	0.24[b]	0.40[ab]	0.74[a]	0.28[b]	0.052	0.014	0.835	0.078
	Veillonellaceae		1.81	1.41	1.68	1.72	2.65	0.199	0.345	0.910	0.152
		Dialister	0.93[ab]	0.44[b]	0.88[ab]	0.76[ab]	1.93[a]	0.168	0.048	0.864	0.054
		Mitsuokella	0.26	0.07	0.28	0.12	0.31	0.050	0.464	0.597	0.742
	Erysipelotrichaceae		2.89	1.84	1.91	2.41	2.01	0.274	0.759	0.252	0.588
		Bulleidia	0.37	0.48	0.62	0.80	0.57	0.095	0.697	0.325	0.524
		Solobacterium	0.55	0.36	0.25	0.39	0.43	0.079	0.849	0.367	0.573

		Alloprevotella	0.35	0.04	0.10	0.41	0.21	0.062	0.270	0.319	0.085
		SR1 genera incertae sedis	0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275
	Acidaminococcaceae		2.88	2.79	1.98	2.56	1.92	0.222	0.529	0.339	0.772
		Succiniclasticum	2.39	2.65	1.62	2.10	1.54	0.204	0.350	0.430	0.483
		Acidaminococcus	0.34	0.09	0.34	0.38	0.34	0.047	0.303	0.689	0.178
Spirochaetes			1.48	1.06	0.76	1.72	0.86	0.210	0.562	0.492	0.422
	Spirochaetaceae		1.16	0.95	0.62	1.43	0.67	0.194	0.668	0.642	0.543
		Treponema	0.64	0.69	0.40	0.99	0.42	0.144	0.697	0.972	0.612
		Sphaerochaeta	0.49	0.14	0.17	0.43	0.24	0.064	0.333	0.149	0.208
Actinobacteria			0.49	0.24	0.63	0.44	0.29	0.063	0.295	0.579	0.624
	Coriobacteriaceae		0.48	0.21	0.61	0.33	0.29	0.060	0.223	0.431	0.445
		Olsenella	0.47	0.20	0.58	0.32	0.28	0.059	0.249	0.405	0.498
Fibrobacteres			4.93^{a}	0.75^{b}	1.97^{ab}	2.61^{ab}	1.41^{ab}	0.436	0.028	0.003	0.465
	Fibrobacteraceae		4.93^{a}	0.75^{b}	1.97^{ab}	2.61^{ab}	1.41^{ab}	0.436	0.028	0.003	0.465
		Fibrobacter	4.93^{a}	0.75^{b}	1.97^{ab}	2.61^{ab}	1.41^{ab}	0.436	0.028	0.003	0.465
Tenericutes			0.06	0.14	0.27	0.32	0.19	0.058	0.657	0.262	0.699
SR1			0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275
	SR1 genera incertae sedis		0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275
		SR1 genera incertae sedis	0.19	1.49	0.00	0.00	0.00	0.303	0.452	0.816	0.275
Unclassified			0.47^{b}	1.76^{a}	0.71^{b}	0.98^{ab}	0.61^{b}	0.127	0.006	0.067	0.083
	Unclassified		7.70	15.10	8.54	10.76	7.74	1.072	0.139	0.297	0.267
		Unclassified	20.19^{b}	31.94^{a}	22.40^{b}	21.90^{b}	23.05^{ab}	1.150	0.006	0.085	0.042

SEM: Standard error of mean

[1]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.

[2]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).

Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.

Table 5. Effect of different tannin extracts on relative abundance (%) of methanogen taxa in the rumen of lambs

			Dietary treatment[1]							Contrast[2] (*P*-value)	
Class	Family	Genus	CON	HT-c	HT-t	CT-m	CT-g	SEM	*P*-value	Tannin	Tannin type
Methanobacteria			32.01	25.80	35.99	28.01	32.18	2.548	0.756	0.825	0.891
	Methanobacteriaceae		32.01	25.80	35.99	28.01	32.18	2.548	0.756	0.825	0.891
		Methanobrevibacter	29.39	24.77	33.87	26.40	30.65	2.385	0.781	0.942	0.884
		Methanosphaera	2.62	1.03	2.12	1.61	1.53	0.228	0.244	0.080	0.993
Methanomicrobia			0.03	0.03	0.06	0.03	0.02	0.007	0.306	0.880	0.156
	Methanocorpusculaceae		0.02	0.02	0.05	0.03	0.01	0.006	0.343	0.482	0.451
		Methanocorpusculum	0.02	0.02	0.05	0.03	0.01	0.006	0.343	0.482	0.451
	Methanosarcinaceae		0.02	0.01	0.02	0.00	0.00	0.003	0.510	0.371	0.138
Thermoplasmata			0.95	11.64	1.46	12.00	3.15	1.750	0.085	0.163	0.782
	Methanomassiliicoccaceae		0.94	11.63	1.46	12.00	3.15	1.750	0.085	0.163	0.781
		Methanomassiliicoccus	0.94	11.63	1.46	12.00	3.15	1.750	0.085	0.163	0.781
Unclassified			67.01	62.53	62.48	59.96	64.65	2.757	0.955	0.540	0.975
	Unclassified		67.02	62.54	62.49	59.96	64.65	2.757	0.955	0.539	0.974
		Unclassified	67.03	62.55	62.50	59.96	64.66	2.757	0.955	0.539	0.973

SEM: Standard error of mean

[1]Dietary treatments: CON: Control; HT-c: Chestnut hydrolysable tannin; HT-t: Tara hydrolysable tannin; CT-m: Mimosa condensed tannin; CT-g: Gambier condensed tannin.

[2]Contrasts: Tannin (overall effect of tannin: CON *vs*. HT-c + HT-t + CT-m + CT-g); Tannin type (effect of HT *vs*. CT: HT-c + HT-t *vs*. CT-m + CT-g).

Dietary treatment means within a row with different superscripts differ at a significance of $P < 0.05$.