Agroindustrial by-products from tomato, grape and myrtle given at low dosage to lactating dairy ewes: effects on rumen parameters and microbiota

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ABSTRACT
The inclusion of by-products in composite pellet feeds could be a strategy to recycle nutritional components and a commercial opportunity for feeds industries. Aim of this study was to evaluate if the use of small amount of dried by-products containing polyphenols could alter rumen fermentation parameters and microbiota in dairy ewes. Thirty-six ewes (9 animals per group) were fed control diet (CON), and diets supplemented with 100 g/d per animal of tomato pomace (TP), 100 g/d per animal of grape marc (GM), and 75 g/d per animal of exhausted myrtle berries (EMB). Treatment showed significant effects only on pH, whereas sampling time affected almost all rumen fermentation parameters. No differences in the proportion of any phylum were detected in GM group compared to CON group. TP, compared to CON, evidenced higher abundance of Proteobacteria phylum (counts of 4.86; log fold chain of 5.61) which was dominated by Acetobacteraceae family with Acetobacter genus. The supplementation of EMB resulted in higher abundance of Succinivibrionaceae and lower abundance of Veillonellaceae family. Furthermore, in EMB group the Paraprevotellaceae and Prevotellaceae families dominated the phylum of Bacteroidetes. This phylum was significantly different between EMB and CON diet (counts of 57.06; log fold change of 1.10). The estimated production of methane and rumen fermentation parameters were not affected by the diets. The supplementation in small doses of GM, TP and EMB in the diets of sheep did not evidence a clear variation in rumen fermentation parameters nor changes in the general structure of rumen microbiota.

HIGHLIGHTS
- The agroindustrial by-products did not affect the rumen biochemical parameters.
- The structure of bacteria community was influenced by EMB diet.
- GM and TP diets did not increase rumen ammonia.

Introduction
By-product feeds can be used to maintain acceptable animal performance while decreasing feed costs (Lourenco et al. 2016). Some agro-industrial by-products used in animal diets contain considerable amounts of the bioactive compound, such as polyphenols, that could affect rumen microbiota and rumen fermentation parameters. By-products from the wine-making process (e.g. grape marc, GM) (Spanghero et al. 2009), from the tomato industry (e.g. tomato pomace, TP), or those from the myrtle distillation process (e.g. exhausted myrtle berries, EMB) contain interesting amounts of polyphenols (Chedea et al. 2017; Croreddu et al. 2019; Nudda et al. 2017) or others bioactive phytochemicals, such as sterols, tocopherols, carotenes (Kalogeropoulos et al. 2012). In ruminant diets, polyphenols affect the ruminal microbial populations, inducing community shifts in the bacteria, fungi and protozoa (Patra and Saxena 2009; Buccioni et al. 2015; Buccioni et al. 2017) and causing relevant changes in ruminal metabolism of nutrients (Patra and Saxena 2011; Buccioni et al. 2012; Cappucci et al. 2018). Polyphenols may decrease methanogenesis (Dschak et al. 2011; Liu et al. 2011) and protein degradation in the rumen and increase protein production and protein flow to the duodenum targeting specific groups of rumen microbial populations (Patra and Saxena 2011). Furthermore, the by-products rich in polyphenols included in ruminant diets can reduce ammonia production (Bhatta et al. 2009; Theodoridou 2020).
Tannins can interfere with membrane functions of rumen bacteria, binding enzymes, or inducing depletion of bioavailable iron by stable complexation (Patra and Saxena 2011). The in vivo and in vitro experiments investigating tannin effects on biohydrogenation showed inconsistent results due to the differences between polyphenols types, amount of polyphenols, and their association with other components of the diet (Buccioni et al. 2015; Buccioni et al. 2017). For instance, condensed tannins are not usually toxic to ruminants since they are not absorbed, but they may bind several nutrients irreversibly, making them unavailable to the animal, and they can also reduce the functionality of complex gastrointestinal enzymes (Smeriglio et al. 2017). On the other hand, the hydrolysable tannins may cause toxicity responses to ruminants when consumed at excessive amounts, whereas they could provide beneficial effects when used at low to moderate concentrations (Reed and Tanprasert 1995; Vasta et al. 2019). For example, grape marc used in a small dose in the diet of dairy sheep enhanced milk, protein, and fat yield (Nudda et al. 2017). In ruminants, the feeding approaches to improve the efficiency of energy and protein utilisation and minimise methane emissions and N excretion is strategic to enhance animal performances and reduce environmental impact. Considering the circular economy principles and the EU regulations on the feed use of food no longer intended for human consumption, the inclusion of agroindustrial by-products and food waste into animal diets could represent a solution to valorise and redirect them towards a more efficient re-use. The use of small doses of by-products in dry form could be an interesting resource to feed industries for their potential inclusion in pelleted formulations. This experiment was designed to evaluate if the inclusion of low dosages of by-products, such as grape marc, tomato pomace and exhausted myrtle berries, affects rumen fermentation parameters and ruminal microbiota populations of lactating dairy ewes.

**Materials and methods**

**Animals and diets**

Thirty-six Sarda dairy ewes were randomly allocated into 4 experimental groups (9 animals per group) as detailed in a previous publication (Nudda et al. 2017). Each group received a TMR as a basal diet (control group, CON) supplemented with 100 g of ground grape marc (GM group), 75 g of ground exhausted myrtle berries (EMB group), or 100 g of ground dry tomato pomace (TP group). The TMR was formulated to meet the energy and protein requirements of the ewes as calculated by the Small Ruminant Nutrition Model (Tedeschi et al. 2010). The chemical composition of the diets and of the by-products and the FA and phenol composition of by-products used in this experiment are extensively reported in Nudda et al. (2017) and resumed in Supplemental Table S1.

**Sampling and analysis**

**Rumen liquor sample collection**

An individual sample of rumen liquor was collected on days 30 and 45 of the experimental period from the animals assigned to the 4 experimental groups. Rumen content was sampled 2 h after morning feeding using a stomach tube and an evacuation pump. To reduce saliva contamination, the first 30 mL of the rumen fluid was discarded. The sample of the rumen fluid was immediately filtered through sterile gauze. The pH was recorded after sampling using a pH metre (Orion 250 A, Orion Research Inc., Boston, MA) equipped with a glass electrode (model 238405, Hamilton Company, Reno, NV). Individual rumen liquor was divided in 3 aliquots: 2 tubes (10 mL each), added with 0.2 mL H2SO4 (50%) to acidify the sample, were stored to analyse ammonia and volatile fatty acids (VFA), respectively. Another aliquot (about 250 mL) was collected into a stool stabiliser tube included in the extraction Kit (PSP® Spin Stool DNA Kit) for subsequent DNA analysis.

**Ammonia content and volatile fatty acids determination in rumen liquor**

Ammonia and VFA contents in rumen liquor were determined according to Correddu et al. (2015). Briefly, NH3 content was determined through the colorimetric method, by using a UV-Visible Spectrophotometer (Varian, Inc., Palo Alto, CA) following Chaney and Marbach (1962) by substituting phenol with salicylate. The VFA was determined using a high-performance liquid chromatographic method according to Correddu et al. (2015). The concentration of total and individual VFA was expressed as mmol/L and mol/100 mol of total VFA, respectively.

**Estimation of methane emission**

The methane emission was estimated according to the equation of Moss et al. (2000):
where C₂ is acetate; C₃ is propionate and C₄ is butyrate expressed as mol/100 mol of VFA.

**Microbial analysis**

**DNA extraction**

Total DNA was extracted from 250 μL of rumen liquor following the PSP method commercially available (PSP® Spin Stool DNA kit), already tested on sheep rumen samples (Henderson et al. 2013). Briefly, 1.4 mL of stabilised rumen sample was transferred into a 2-ml safe look tube, incubated for 10 min at 95°C on a thermomixer at 900 rpm; 5 zirconia beads were added to the homogenate, and then the samples were centrifuged at 11,000 × g for 1 min at room temperature. The supernatant was transferred into InvitAdsort-Tube and vortexed vigorously (15 s). The suspension was incubated for 1 min at room temperature, and centrifuged at 11,000 × g for 2 min. The filtrate and the tube were discarded, and the RTA Spin Filter was transferred into a new tube and washed two times with the buffer solutions prepared previously with kit tools. To eliminate any traces of ethanol the tube was centrifuged again. The RTA spin filter was placed in a new tube and added with elution buffer (preheated to 70°C) and centrifuged (11,000 × g for 1 min) two times. The tube with filter (RTA-Spin filter) DNA was discarded and the total DNA was stored at -20°C.

**Molecular analysis of the bacterial population**

The 16S rRNA Metagenomic Sequencing Library was prepared following the manufacturer’s protocol (Illumina Inc., San Diego, CA). Briefly, samples were amplified in the V3 and V4 regions using denaturated primers (Klindworth et al. 2013) in a limited cycle PCR, followed by an AMPure XP bead clean-up (A63880; Beckman Coulter Inc., Brea, CA). A second PCR reaction was then performed to attach dual index and Illumina sequencing adapters using the Nextera XT Index Kit, followed by a final AMPure XP bead clean-up. Finally, library concentration was measured by fluorometric quantification using Qubit 2 (Invitrogen Inc., Carlsbad, CA) and each library was validated using a 1:50 dilution of the library on a Bioanalyzer DNA 1000 chip to verify sample size. Calculate DNA concentration in nM, based on the size of DNA amplicons, was used to pool the obtained 72 libraries in equimolar concentration. Pooled library was sequenced using the Illumina Miseq technology in 2 × 300 bp run with 5% of PhiX library as control. The sequences of raw data were filtered out and the reads were trimmed to a consistent length. Then, the data was denoised, chimera filtered, and taxonomically assigned using DADA2 v1.1.5 (Callahan et al. 2016). For the taxonomic analysis, the sequencing reads were clustered into operational taxonomic units (OTUs) defined as groups of sequencing reads that differ by less than a fixed dissimilarity threshold (97%) generated in DADA2 using the Greengenes database v13.5 (McDonald et al. 2012).

**Statistical analysis**

**Rumen parameters**

Data of rumen parameters (AGV, pH, NH₃ and estimated methane production) were analysed with the PROC MIXED procedure of SAS (2002). The following mixed linear model was adopted:

\[
y_{ijk} = \mu + \text{diet}_i + \text{date}_j + \text{diet}_i \times \text{date}_j + \text{animal}_k(\text{diet}_i) + e_{ijk}
\]

where \(y_{ijk}\) is the rumen parameter; \(\mu\) is the overall mean; \(\text{diet}_i\) is the fixed effect of \(i\)-th dietary treatment (CON, TP, EMB and GM); \(\text{date}_j\) is the fixed effect of the \(j\)-th sampling date (S1 and S2); \(\text{diet}_i \times \text{date}_j\) is the interaction between \(i\)-th diet and \(j\)-th sampling date; \(\text{animal}_k(\text{diet}_i)\) is the random effect of the \(k\)-th animal nested within the \(i\)-th dietary treatment; \(e_{ijk}\) was the random residual error. Means were separated using the Tukey test and significance was declared at \(p < .05\).

**Microbial analysis**

Microbial data were analysed using the software packages from the open-source Bioconductor project (Callahan et al. 2016). All data was collected into a phyloseq object (McMurdie and Holmes 2013) and used for further exploratory analysis. Briefly, alpha diversity (within-sample diversity) was assessed using Simpson and Shannon metrics on inverse Simpson diversity index (Simpson 1949; Hill 1973) and Shannon diversity index (Shannon and Weaver 2001).

Beta diversity (between-sample diversity comparison) was assessed with non-metric multidimensional scaling (NMDS) chosen to represent the dissimilarity between samples in a low-dimensional space based
on Bray–Curtis dissimilarity matrix. Variance stabilising transformation (McMurdie and Holmes 2014) was used for 16S rRNA generated count data using DESeq2 package (Love et al. 2014). Each microbial group was tested individually, measuring its association with sample diet, and then jointly adjust p-values to ensure a False Discovery Rate (FDR) through the Benjamini–Hochberg procedure (Benjamini and Hochberg 1995).

Results

Rumen pH, VFA, methane and ammonia

The results of the effects of EMB, TP and GM on the concentration of total VFA, molar proportions of individual VFA, acetate:propionate ratio, NH3 and estimated methane production are presented in Table 1.

The ruminal pH ranged between 6.30 and 6.44 and it was not different between by-products diets and the CON one, whereas among by-products TP showed a lower pH compared to GM. All other parameters were not significantly influenced by any by-products compared among them and to CON indicating that, at the dosage utilised in this experiment, their polyphenolic compounds did not interfere on ruminal biochemistry. Time of sampling affected almost all rumen parameters, except for acetate mol incidence, propionate percentage and methane estimated emissions.

Regarding the interaction sampling time × treatment, only rumen ammonia content was significantly different (p < .01; Figure 1). The rumen NH3 concentration did not change in GM and TP groups between the first and the second sampling, whereas increases in CON and EMB groups. Hydrolysable tannins followed by ellagic acid and gallic were the main components in EMB (Supplemental Table S2). The analysis of TP in our trial allowed the identification of narigenin and rutin as the major phenolic constituents (Supplemental Table S2). The main phenolic compounds in GM were gallic acid, catechin and anthocyanins (Supplemental Table S2).

Rumen bacterial population

The effects of the dietary by-products on the alpha diversity of rumen bacteria are shown in Table 2. The Shannon index, which measures the diversity of the bacterial communities, did not revealed difference between any by-product and the CON group; whereas significant differences between EMB and GM were evidenced (p < .05) The Shannon index of bacteria did not change between sampling time for any treated group. The Simpson’s index was significantly higher for EMB compared to GM at sampling 1, but any group receiving by-product differs from CON.

In terms of beta diversity, nonmetric dimensional scaling (NMDS) revealed statistical difference in the microbiota between sampling time (Figure 3). Total genus abundance grouped by diet and separate for sampling time (Supplemental Figure S3(a)), and the

Table 1. Effects of dietary treatments on rumen parameters.

| Diet1 | pH | Ammonia, mg/dL | Acetate | Propionate | Butyrate | Isovaleric | Valeric | Acetate/Propionate | Propionate, % | Butyrate, % | CH4 emission4,5 | CH4/kg of DM5 | CH4/kg of milk5 |
|-------|----|----------------|---------|------------|----------|------------|---------|------------------|--------------|------------|----------------|----------------|----------------|
| CON   | 6.36ab | 6.40ab | 6.26b   | 6.44a | 0.025    | NS            | NS      | NS               | NS           | NS         | NS             | NS             | NS             |
| EMB   | 6.40ab | 6.40ab | 6.26b   | 6.44a | 0.025    | NS            | NS      | NS               | NS           | NS         | NS             | NS             | NS             |
| TP    | 6.26b  | 6.26b  | 6.26b   | 6.44a | 0.025    | NS            | NS      | NS               | NS           | NS         | NS             | NS             | NS             |
| GM    | 6.44a  | 6.44a  | 6.44a   | 6.44a | 0.025    | NS            | NS      | NS               | NS           | NS         | NS             | NS             | NS             |

1CON: control diet; EMB: diet containing 75 g/d per head of exhausted myrtle berries; TP: diet containing 100 g/d per head of tomato pomace; GM: diet containing 100 g/d per head of grape marc; 2D: effect of diet; S: effect of sampling time; NS indicates p > .1000; 3VFA: volatile fatty acids; 4Estimated according to Moss et al. (2000); 5CH4: Methane, mol/mol of VFA.

Figure 1. Temporal evolution of rumen NH3 production during the trial.
Log transformed abundance and rank coloured by diet (Supplemental Figure S3(b)) confirm the differences only between sampling time.

The Bacterial Community Structure is reported in Tables 3–5. Table 3 shows the top 10 bacterial families or genera ranked for DESeq2 results in EMB compared to CON diet, whereas the remaining taxa were not listed.

The rumen microbiota for the EMB versus CON diet was dominated by OTUs assigned to the Bacteroidetes, Proteobacteria, Cyanobacteria and Firmicutes phylum. The proportion of sequences assigned to the Bacteroidetes, Proteobacteria, and Cyanobacteria phyla was higher, whereas Firmicutes was lower in the EMB than CON group. In particular, supplementation of EMB resulted in higher abundance of Succinivibrionaceae family. Differences in OTU abundances were also detected for the Veillonellaceae family (Table 3), whereas the Paraprevotellaceae and

### Table 2. Diet’s effect on biodiversity indices of rumen microbial population.

| Index    | Sampling | CON  | EMB  | TP   | GM   | SEM^2 | D   | S   | D × S |
|----------|----------|------|------|------|------|-------|-----|-----|-------|
| Shannon  | 1        | 2.48 | 3.02 | 2.38 | 2.23 | 0.06  | **  | NS  | NS    |
|          | 2        | 2.48 | 2.65 | 2.59 | 2.23 | 0.05  |     |     |       |
| Simpson  | 1        | 0.76 | 0.86 | 0.74 | 0.71 | 0.01  | *   | NS  | NS    |
|          | 2        | 0.76 | 0.79 | 0.80 | 0.70 | 0.01  |     |     |       |

^abMeans within a row with different superscripts are different (p < .05).

1CON: control diet; EMB: diet containing 75 g/d per head of exhausted myrtle berries; TP: diet containing 100 g/d per head of tomato pomace; GM: diet containing 100 g/d per head of grape marc; 2SEM: standard error of the mean; 3D: effect of diet; S: effect of sampling time; NS indicates p > .05.

*a,bMeans within a row with different superscripts are different (p < .05).

Figure 2. Violin plot of alpha diversity as measured with different metrics. Different colours indicate different diet treatments, violin shapes represent all possible results, with thickness indicating density. No significant differences at p < .01 were observed between groups.
Prevotellaceae families dominated the phylum Bacteroidetes (Table 3).

The comparison of GM with CON diet did not evidenced differences in the proportion of any phylum between the two groups.

Results of TP compared to CON group, evidenced the higher abundance of Proteobacteria phylum (Table 5). This phylum was dominated by Acetobacteraceae family with the genus Acetobacter.
sheep (Correddu et al. 2019) and was not influenced within the normal range for rumen liquor pH in Sarda microbial population. Values of ruminal pH were affect rumen function, protein and fibre digestion and The inclusion of by-products in ruminant diets can

**Discussion**

The inclusion of by-products in ruminant diets can affect rumen function, protein and fibre digestion and microbial population. Values of ruminal pH were within the normal range for rumen liquor pH in Sarda sheep (Correddu et al. 2019) and was not influenced by treatments compared to the control. No differences were observed, also in the molar proportion of rumen VFA among treatments, in line with previous findings in sheep diet by using both condensed (Hervás et al. 2003), and hydrolysable tannins (Liu et al. 2011). In addition, gallic acid one of the main components of HT, has been found to reduce NH3 in beef (Aboagye et al. 2019). Several rumen microorganisms, have the ability to degrade gallic acid to acetate and butyrate (Vasta et al. 2019), and this could be one of the reasons of the ineffectiveness of EMB to affect protein degradation. However, a recent in vitro study showed that the abundance and the efficiency of rumen microorganisms able to reduce gallic acid are very low in rumen liquor (Mannelli et al. 2019) and therefore, others dietary components may have interfered. Moreover, the pattern of NH3 in TP group does not agree with previous observation in beef cattle where rumen ammonia increases with the inclusion of dried tomato, as an alternative to soybean meal, in a dose (2 g of dried tomato pomace per kg of body weight; Yuangklang et al. 2010) similar to the one used in our experiment (2.2 g/kg of body weight; Nudda et al. 2017). Therefore, the discrepancy in the results could be related to the species-specific differences and/or to basal diet.

Data about the microbial population of rumen fluid showed that the supplementation of EMB evidenced higher abundance of *Succinivibrionaceae* family,
Veillonellaceae family, and Paraprevotellaceae and Prevotellaceae families dominated the phylum Bacteroidetes. These bacteria have been reported to contain urease genes and exert urease activity (Patra and Aschenbach 2018), and they have been associated to low methane emissions in Tammar wallabies (Pope et al. 2011). Veillonellaceae family are considered involved in the production of propionate as its major fermentation product (Kishimoto et al. 2006). The TP group had higher abundance of Proteobacteria phylum, dominated by Acetobacteraceae family with the genus Acetobacter, which has been found positively correlated with CH4 emissions in dairy heifers (Cunha et al. 2019). This could explain the tendency for highest CH4 emission per kg of DMI and per kg of milk observed in TP group. Differences in the rumen microbiota between sampling could reflect the variation of numbers and diversity of microbial population according to the changes in dry matter intake (Carberry et al. 2012), that in our study decrease significantly from the first to the second sampling (1.78–1.66 kg/d of DM, respectively; p < .05). The lower dry matter intake could be reflecting the evolution of energy requirement during the natural evolution of the lactation curve of Sarda dairy ewes (Pulina et al. 2007).

Conclusions
The inclusion of low doses of by-products rich in polyphenols in the diets of dairy sheep did not cause consistent change in rumen parameters nor variations in the structure of rumen microbiota in comparison to CON diet. Moreover, the by-products used in the low dose in the trial, did not evidence any effects on estimated methane emitted by in dairy sheep. Almost all biochemical and physiochemical parameters and the structure of the microbiota showed a clear difference between sampling time, related to the evolution of feed intake during the experiment. Finally, the use of these by-products at low dosage did not evidence any negative effects on rumen bacteria or animal performance, suggesting their potential use in commercial feed.

Ethical approval
The data used in the present study were obtained from an experiment carried out according to the Italian laws on animal experiments and ethics (LD 04/03/2014, n.26).

Author contributions
G. Buffa conducted the experiment as a part of her PhD; N. P. Mangia and D. Licastro conducted and interpreted the microbiological part of this experiment; A. Cesarani and S. Sorbolini elaborated the data; G. Pulina reviewed the paper; A. Nudda conceived and wrote the paper. The responsibility for what is written in this paper rests with all authors.

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