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Changes in the solid-associated bacterial and fungal communities following ruminal *in vitro* fermentation of winery by-products: aspects of the bioactive compounds and feed safety

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ABSTRACT

Objectives: Feeding winery by-products (WBP) could affect the bovine microbiome because of their phenol compounds and a transfer of WBP-associated microbiota. This work examined changes in the underexplored solid-associated rumen microbiome following the inclusion of WBP.

Methods: Using the rumen simulation technique, fermenters were inoculated with the inoculum of donor cows and were fed one of six dietary treatments including a control diet of 70 % hay +30 % concentrate (CON), control diet + 3.7 % commercial grapeseed extract (EXT), 65 % hay + 25 % concentrate + 10 % grape pomace (GP-low), 56 % hay + 24 % concentrate + 20 % grape pomace (GP-high), 70 % hay + 25 % concentrate + 5 % grapeseed meal (GS-low), and 65 % hay + 25 % concentrate + 10 % grapeseed meal (GS-high) (dry matter basis). The compositional changes of bacteria, archaea and fungi in the solid fractions were based on 16S and ITS2 rRNA sequencing.

Results: The alpha- and beta-diversity of the microbiota were unaffected. However, treatment modified the bacterial composition at low taxonomic levels. *Butyrivibrio fibrisolvens*, *Treponema bryantii*, and bacterium MC2010 decreased in EXT, while *Treponema berlinense* was increased in GP-high and GP-low compared to CON. Concerning fungi, GS-high increased *Candida* spp., *Lachancea* spp., *Microdochium* spp., *Mucor* spp., *Pichia* spp., *Saturnispora* spp., and *Zygosaccharomyces* spp. compared to CON. Many non-*Saccharomyces* yeasts were detected in WBP samples but absent in donor cows and CON samples. The genera affected by treatment were not the major contributors to the ruminal degradation of nutrients.

Conclusions: The results indicate a sensitivity of rumen solid bacteria to grape phenols when delivered as an extract and a transfer of WBP-associated microbiota into the rumen.

1. Introduction

Rumen microbes convert low-quality, fiber-rich materials into useable energy sources [1]. The complex symbiosis of rumen microorganisms e.g., protozoa, bacteria, viruses, fungi, and archaea that interact and compete for substrates is critical to the balance between nutrients used for microbial growth, short chain fatty acid (SCFA) production, and other beneficial end products for the host [1]. The particle-adherent rumen microbiota composes up to 80 % of the total microbial population in the rumen [2] and is known to be compositionally different and

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Abbreviations: ASV, amplicon sequence variants; DM, Dry matter; GP, Grape pomace; GS, Grapeseed meal; RUSITEC, Rumen simulation technique; WBP, Winery by-products.

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less diverse than its liquid counterpart [3,4]. Fungi and bacteria digest plant polymers into monomers that are then catabolized into SCFA, gas, and alcohols [1], while methanogenic archaea convert H_2 and CO_2 or acetate into CH_4. Some relationships between rumen microbes are synergistic, whereas others are antagonistic [5]. For instance, fungi produce H_2 that can be used as a substrate for methanogenic archaea and may compete with bacteria for the digestion of plant materials [6].

A large body of research exists on ruminal bacteria and archaea whereas ruminal fungi have been less researched [7]. Fungi have significant shares (5–20 %) in the rumen and have essential roles in fiber digestion as they harbor essential enzymes for the degradation of plant materials such as cellulase, xylanase, and hydrolases [8,9]. Moreover, rumen fungi produce hyphae that penetrate recalcitrant (lignified) plant structures [2], easing the access of bacteria into plant cells. The role of fungi in the rumen could be increasingly important when feeding lignified fiber sources such as wine by-products (WBP) like grapeseed meal and grape pomace.

Using the rumen simulation technique (RUSITEC) system, we previously demonstrated the benefit of WBP in decreasing excessive protein degradation that can be related to the effect of their phenolic compounds [10]. A high grape phenol content (about 3 % of diet dry matter (DM), which was equivalent to 20 % of WBP in the diet) affected the community of liquid-associated microbiota (bacteria and archaea). However, in that study, the effects of treatments on nutrient degradation could not be explained by the liquid microbes. Not only does the composition of solid adherent bacteria differ from those in liquid fractions [11] but solid communities also include denser populations of anaerobic fungi [12] performing important roles in the rumen of ruminants [13]. Thus, solid microbiota may provide meaningful information supporting nutrient degradation data. In this work, we further investigated the stability of the feed-adherent (solid) microbial consortium, including bacteria, archaea, and fungi after ruminal *in vitro* incubation of WBP. Studying solid communities, particularly the mycobiota, offers the opportunity to investigate the transfer of feed particle-associated microorganisms into the rumen ecosystem. This aspect is of significance when feeding by-products from fermented foods like WBP, in which the outcome can be considered neutral, positive (e.g., supplying probiotic bacteria), or negative (spoilage microbes such as molds and yeasts). For instance, microbiological analyses of grape pomace showed counts of aerobic mesophilic bacteria, lactic acid bacteria, spore-forming bacteria, yeasts, and even some potential pathogens like *Enterobacteriaceae* and *Clostridium perfringens* [14].

To study the role of WBP on the solid microbiota (bacteria, archaea, and fungi), 16S rRNA and ITS2 sequencing were conducted in the present study. To identify microbiota unique to WBP, samples of donor cows used for inoculating the RUSITEC system were also investigated. We expected shifts in the composition of solid consortium when including WBP because of the presence of grape phenols as well as a transfer of WBP-associated microbiota into the rumen community. The current research offered novel microbiological insights concerning the role of bioactive compounds as well as a feed safety aspect in ruminant production when using by-products from food production.

2. Materials and methods

2.1. Experimental design and treatments

The experiment was carried out using two RUSITEC systems, each with six experimental units, yielding a total of 12 units per experimental run (Fig. 1). In total, four experimental runs were conducted. Each experimental run lasted 10 days, where the first 5 days were used as an equilibration of the system before sampling. All experimental diets contained high-quality hay and grain mix (i.e., concentrate) as basal ingredients. The contents of hay and concentrate varied according to the substitution with grapeseed meal or grape pomace. Specifically, the control diets consisted of 70 % hay +30 % concentrate (CON) on a DM basis. The positive control diet, EXT, consisted of the control diet + commercial grapeseed extract (EXT, Nature Love® OPC Grape Seed Extract, Tauron Ventures GmbH, Düsseldorf, Germany) at 3.7 % of the CON diet DM. WBP treatment groups included a diet with 56 % hay +24

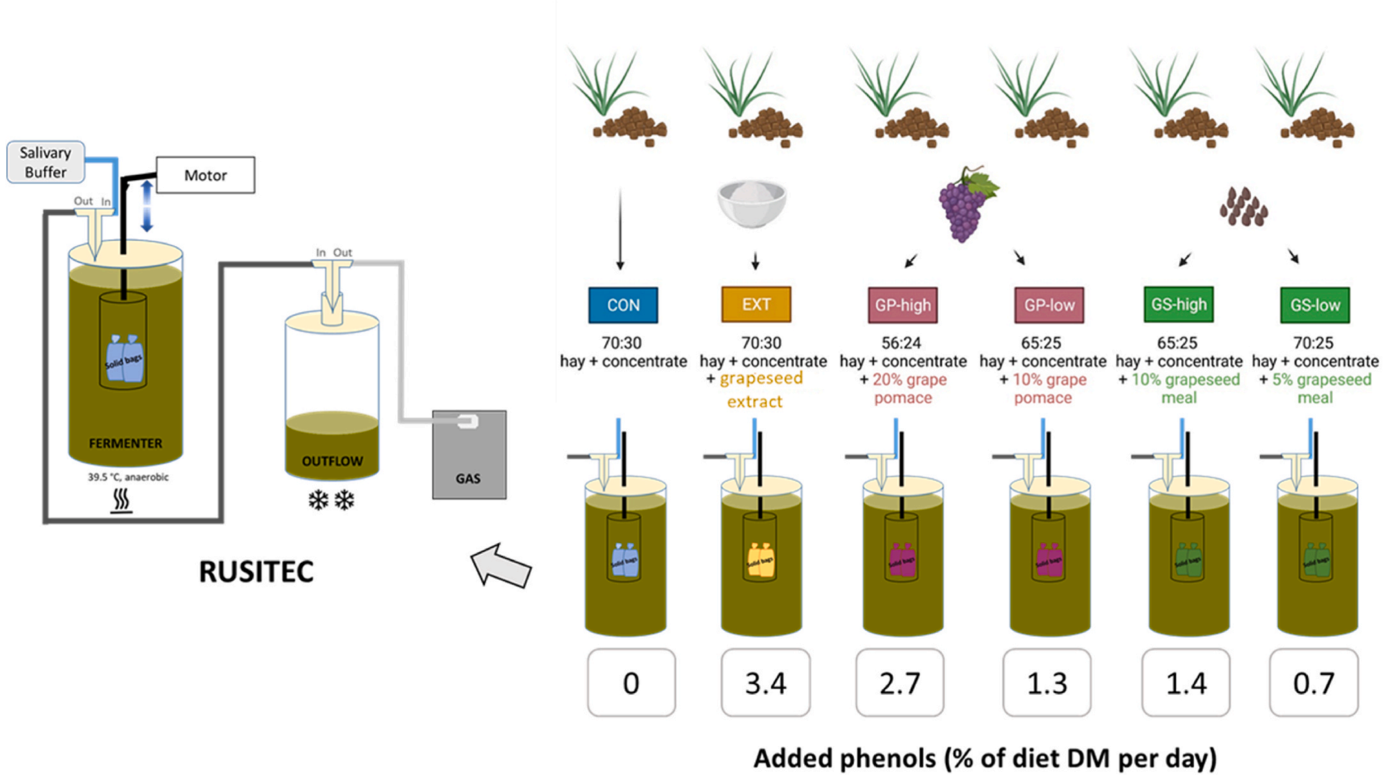

Fig. 1. Schematic illustration of dietary treatments and the rumen simulation technique (RUSITEC) system.

% concentrate +20 % grape pomace (GP-high), 65 % hay + 25 % concentrate + 10 % grape pomace (GP-low), 65 % hay + 25 % concentrate + 10 % grapeseed meal (GS-high), and 70 % hay + 25 % concentrate + 5 % grapeseed meal (GS-low), also on a DM basis. The substitution scheme of WBP intended to keep similar nutrient chemical compositions and forage to concentrate ratios of all diets as much as possible while including the WBP dosages according to previous test dosages and recommendations [15,16].

The hay was mainly a mixture of first- and second-cut *Lolium perenne*. It contained, on a DM basis, 6.84 MJ/kg net energy lactation, 91.7 % organic matter, 21.9 % crude protein; 20.5 % water soluble carbohydrates, 45.2 % neutral detergent fiber, and 2.2 % ether extract. The grain mix consisted of 21.6 % barley; 21.6 % wheat; 51.7 % corn; and 5.2 % vitamin and mineral supplement (Rindavit TMR 11 ASS-CO + ATG; H. Wilhelm Schaumann GmbH & Co KG, Brunn/Gebirge, Austria) on a DM basis. WBP was obtained from a local producer of red wine in the Gumpoldskirchen, Austria. Ingredients were ground with a Wiley mill (Pulverisette 25/19, FRITSCH GmbH, Idar-Oberstein, Germany) to pass through a 6-mm sieve. Further information about pH, redox potential, SCFA, nutrient degradation, and microbiota composition in the liquid fraction is given in our companion paper [10].

2.2. RUSITEC procedure

Rumen contents were obtained from two non-lactating rumen fistulated Holstein cows kept at the University of Veterinary Medicine, Vienna. Donor animals were kept on straw bedding and fed hay *ad libitum* with a daily allowance of 0.5 kg of commercial concentrate (KuhKorn PLUS Energie, Garant-Tiernahrung GmbH, Pölchlarn, Austria). The collection of rumen contents was done approximately 3h after the morning feeding and separated into solid and liquid contents, with the latter being strained using four layers of medical gauze (1 mm pore size). Liquid contents were then pooled and used to inoculate the RUSITEC system. RUSITEC is a semicontinuous *in vitro* model widely used to study ruminal fermentation, allowing the detection of quantitative and qualitative changes in microbial populations in a standardized environment [17]. The RUSITEC procedure was performed as previously described [10]. Briefly, each fermenter was inoculated with 600 ml of rumen fluid and 100 ml of pre-warmed McDougal's buffer [18]. The fermenters were continuously infused with the buffer using a 12-channel peristaltic pump (model ISM932, Ismatec, Idex Health & Science GmbH, Wertheim, Germany) at a rate of 375 ml/d throughout the experiment. Temperature was maintained at 39.5 °C through thermostatically controlled water baths. Anaerobic conditions of the system was enabled via flushing with a stream of $N_{2.}$ On the first day of each experimental run, two nylon bags (140 × 70 mm, 150 μm pore size, Fa. Linker Industrie-Technik GmbH, Kassel, Germany) were added to each fermenter. One bag contained pooled solid contents from the donor cows, while the second bag contained 12 g DM of the experimental diet. After 24 h of incubation, the bag containing the mixed solid contents from the donor cows was replaced with a new bag containing the experimental diet. On the next day, the older feed bag, which remained inside the fermenter for 48 h, was replaced with a fresh one. The feedbag exchange procedure was repeated daily.

The RUSITEC fermentation led to degradation (% of supply) of about 61–65 % for DM, 59–63 % for organic matter, 69–71 % for crude protein, and 40–47 % for neutral detergent fiber. In addition, fermentation efficiency was estimated from the production of a fermentation product normalized by the amount of degraded nutrients. Specifically, the production of short-chain fatty acids ranged from 8.9 to 9.6 mmol per gram of degraded organic matter, CH_4 from 11.7 to 12.3 ml per gram of degraded organic matter, and ammonia from 4.0 to 4.9 mmol per gram of degraded crude protein. For more details, please refer to Khiaosa-ard et al. [10].

2.3. Isolation of solid-associated microbes, DNA extraction, and next-generation sequencing

The original rumen solids of individual donor cows (a total of n = 6) and the day-10 feed bag contents obtained from three experimental runs (a total of n = 6 per treatment) were snap-frozen in liquid nitrogen and stored at −20 °C until microbial analysis. Total microbial DNA was isolated from 250 mg of solid content of each feed bag using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) with some modifications following the method described previously [19]. Briefly, samples were added to bead beating tubes and mixed with solution C1, followed by heat (incubation at 95 °C for 5 min), chemical (100 μl of 100 mg/ml lysozyme, 10 μl of 2.5 U/ml mutanolysin (Sigma Aldrich, Vienna, Austria), 21 μl of 18.6 mg/ml proteinase K (Sigma Aldrich)) and mechanical disruption (bead-beating using the FastPrep-24 instrument (MP Biomedical, Santa Ana, CA, USA). Subsequently, sequential centrifugation steps with the solutions C2 - C5 provided by the kit (each step with 15,000×*g* for 1 min) were performed following the manufacturer's instructions, which allowed further removal of cell debris and PCR inhibitors. The supernatant was transferred to new tubes and DNA was eluted in 100 μl of solution C6 followed by centrifugation at 15,000×*g* for 1 min. Measurement of total DNA was performed using the Qubit Fluorometer 2.0 (Qubit dsDNA HS Assay Kit, Thermo Fisher Scientific, Vienna, Austria) according to the manufacturer's instructions. A total volume of 40 μl of each DNA sample was sent for amplicon sequencing of 16S and Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) using Illumina MiSeq paired-ends sequencing technology (Microsynth AG, Balgach, Switzerland). For bacteria and archaea, targeted amplification of the hypervariable region V4 of the 16S rRNA genes (2 × 250 bp) was performed using the universal primers 515F (5′-GTGCCAG CMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [20]. The eukaryotic ITS2 region was sequenced using the primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGA TATGC-3′) [21,22]. Multiplexed libraries were constructed by ligating sequencing adapters and indices onto purified PCR products using the Nextera XT Sample Preparation Kit (Illumina, Balgach, Switzerland). After trimming adapters and primers, corresponding overlapping paired-end reads were stitched by Microsynth (Microsynth AG).

2.4. Read processing and bioinformatics analysis

For bacterial data, merged reads were processed using the software package Quantitative Insights into Microbial Ecology (QIIME2 v2020.2) (https://qiime2.org) and respective plugins [23]. Preliminary read quality was assessed using FASTQC v. 0.11.5 [24]. Sequences were further quality filtered using the q-score-joined plugin with a minimum acceptable PHRED score of 20 (–p-min-quality 20). All reads were trimmed to a length of 250 nucleotides and denoised into amplicon sequence variants (ASVs) with Deblur [25]. Features with an abundance below 10 were removed from the analysis. Representative sequences and feature table were filtered to exclude all features classified as mitochondria or chloroplast sequences. The resulting features were aligned with mafft [26] and used to construct a phylogenetic tree with fasttree2 [27]. Taxonomy was assigned to ASVs using a classify-sklearn naive Bayes taxonomy classifier trained on the 515F/806R primer set against the SILVA 132 99 % OTUs reference sequences (https://www.arb-silva.de, version 132). Rooted tree, taxonomy file, and filtered feature table were used as an input to the R [28] package phyloseq v1.34.0 [29] in RStudio [30]. For ITS data, the forward reads were quality-filtered in the same way as the first dataset. Due to extremely low quality, reverse reads were discarded. Denoising was performed with Deblur [25] with all sequences trimmed to a length of 200 nucleotides and keeping features with an abundance above 3. Taxonomy was assigned to fungal ASVs using a classify-sklearn naive Bayes taxonomy classifier trained on the full length of the ITS sequence using the UNITE database. Unclassified features at the phylum level were removed from downstream analysis. All samples with <1000 features were discarded, and no rarefaction was performed following the recommendations from Beiko et al. [31].

2.5. Statistical analysis

Alpha-diversity indices (number of observed ASVs, Shannon, inverse Simpson, and Fisher's alpha indices) were calculated in phyloseq v1.34.0. The normality of data was visually inspected using both density and Q-Q plots and tested using the Shapiro-Wilk test ($P < 0.05$). Differences in community richness and diversity were analyzed with the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) using a model with treatment as fixed effect and experimental run and fermenter as random effects. Contrasts between CON vs GP and CON vs GS were calculated. Significance was declared at $P \leq 0.05$ and a trend was set at $0.05 < P \leq 0.10$. The results of the mixed models are reported as least squares (LS) means ± standard error of the mean (SEM). Analyses of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) were calculated using Aitchison (clr-transformed) and Bray–Curtis distance matrices. Canonical correlation analysis (CCA) was performed to identify associations between distance matrices and treatments. Comparisons of the microbial abundances among treatments at family and genus levels were performed using MaAsLin2 [32] v1.8.0 using default settings. The false discovery rate [33] corrected P-values ≤ 0.05 (Q-value) were considered to indicate significant differences. Spearman correlations between genera and parameters associated with nutrient digestibility were calculated using Hmisc v4.5-0. Only correlations with $r > 0.6$ or $r < -0.6$ and a P-value <0.05 were further considered.

3. Results

3.1. Sequencing results

A total of 42 solid samples were used for both 16S and ITS2 rRNA sequencing (6 samples from the two donor cows and 36 experimental samples). Sequencing of the V4 hypervariable region yielded a total of 2,275,893 merged reads with a median length of 253 bp. After quality filtering, 3217 reads were excluded from the analysis. Denoising with Deblur, removal of mitochondria, chloroplast contaminants, and low abundant features resulted in a total of 1,638,939 sequences and 4322 features. From these, 4290 features were classified as Bacteria and 32 as Archaea (phylum Euryarchaeota). ITS2 sequencing resulted in 1,009,142 forward reads with a median of 225 bp. Quality filtering resulted in a final amount of 971,256 reads. After denoising, the removal of low abundant features, a total of 427,131 sequences and 353 features were retained for downstream analysis. After the removal of unclassified ASVs at the kingdom and phylum level and the exclusion of samples with <1000 features, one sample of EXT was removed, and 349 fungal features were kept in the analysis.

3.2. Community richness, diversity, and phylum composition of solid adherent microbiome

No effects of treatment on alpha diversity were detected ($P > 0.1$) for either fungi or bacteria and archaea (Table 1). When running contrasts between CON, EXT, GS, and GP, a higher amount of observed fungal ASVs was found for GS when compared with CON ($P = 0.05$).

Beta-diversity of fungi tended to be affected by treatment according to the Aitchison distances (PERMANOVA $P = 0.09$, ANOSIM $P = 0.10$), and a distinct separation between EXT and GP treatments from CON was observed in the CCA (Fig. 2). A permutation test showed a significant effect of treatment as an explanatory variable in the CCA model ($P = 0.017$). Beta-diversity of bacteria and archaea was unaffected by treatment according to the Aitchison distances (PERMANOVA $P = 0.17$, ANOSIM $P = 0.16$).

In total, there were 5 fungal phyla and 18 bacterial and archaeal phyla identified in the samples. In the donor cow samples, Neocallimastigomycota was about 96.20 % of the total mycobiota, followed by Mucoromycota (2.38 %) and Ascomycota (1.31 %), while other phyla composed less than 1 % of the relative frequency of fungi (Supplementary Fig. 1). There was a decrease in the percentage of Neocallimastigomycota at the expense of Ascomycota and Mucoromycota ($P < 0.05$). Among RUSITEC samples only, the percentages of Ascomycota and Mucoromycota were affected by treatment. The majority of the reads of bacterial and archaeal phyla were assigned to Firmicutes, Bacteroidetes, Spirochaetes, Actinobacteria, Proteobacteria, Tenericutes, Fibrobacteres, Kiritimatiellaeota and Patescibacteria (Supplementary Fig. 1). There was a shift in the compositions of the donor cows compared with RUSITEC samples but there was no difference in phylum composition among treatments.

3.3. Differential abundance at lower taxonomical levels impacted by treatment

At lower taxonomic levels (family to species), EXT and GS-high consistently impacted fungal composition. Several fungal genera were increased in GS-high compared to CON ($Q < 0.05$) and fewer genera were increased in EXT ($Q < 0.05$) (Fig. 3). *Sporobolomyces* was consistently decreased by EXT, GP-low, and GS-low ($Q < 0.05$). At the species level, in total, ninety fungal species were identified across all samples (detailed data not shown). The highly abundant unidentified species of *Neocallimastigaceae* was suppressed by EXT ($Q < 0.05$) and GP-high ($P < 0.10$) (Fig. 4). Ten fungal species were affected by treatment ($Q = 0.05$, Supplementary Fig. 2), and six other species showed a tendency of change ($0.5 < Q \leq 0.10$). Many of these affected species were minor in

Table 1
Alpha-diversity indices of solid-associated bacteria, archaea and fungi following provision of diets with or without winery by-products.[a]

Item	Treatment						SEM	P -value	P-value Contrasts		
	CON	EXT	GP-low	GP-high	GS-low	GS-high			CON vs. EXT	CON vs. GP	CON vs. GS
Bacteria/Archaea											
Number of observed ASVs	627.33	597.50	562.91	621.35	567.33	716.17	43.18	0.11	0.59	0.47	0.76
Shannon index	4.28	4.06	4.41	4.21	4.26	4.31	0.19	0.21	0.10	0.74	0.96
Inverse Simpson	28.33	23.25	32.94	26.82	26.95	27.51	7.08	0.48	0.25	0.69	0.77
Fisher's alpha	106.55	98.02	98.22	104.76	98.73	122.34	7.44	0.06	0.32	0.50	0.59
Fungi											
Number of observed ASVs	53.50^{b}	56.39^{b}	57.76^{ab}	62.67^{ab}	61.32^{ab}	73.17^{a}	10.87	0.20	0.72	0.33	0.05
Shannon index	1.96	1.81	2.19	1.94	1.94	2.16	0.13	0.29	0.41	0.49	0.55
Inverse Simpson	5.01	3.87	6.39	4.84	4.84	5.63	0.64	0.21	0.23	0.45	0.77
Fisher's alpha	7.69	8.20	8.06	8.80	8.60	10.60	1.70	0.24	0.69	0.49	0.07

[ab]Values sharing no common superscripts differ significantly ($P < 0.05$).

[a] Diets included hay and concentrate mix without (CON) or with different inclusion levels of grape pomace (GP: GP-low = 10 %, GP-high = 20 %), grapeseed meal (GS: GS-low = 5 %, GS-high = 10 %) or top-dressed with grapeseed extract at a 3.4 % of diet dry matter (EXT).

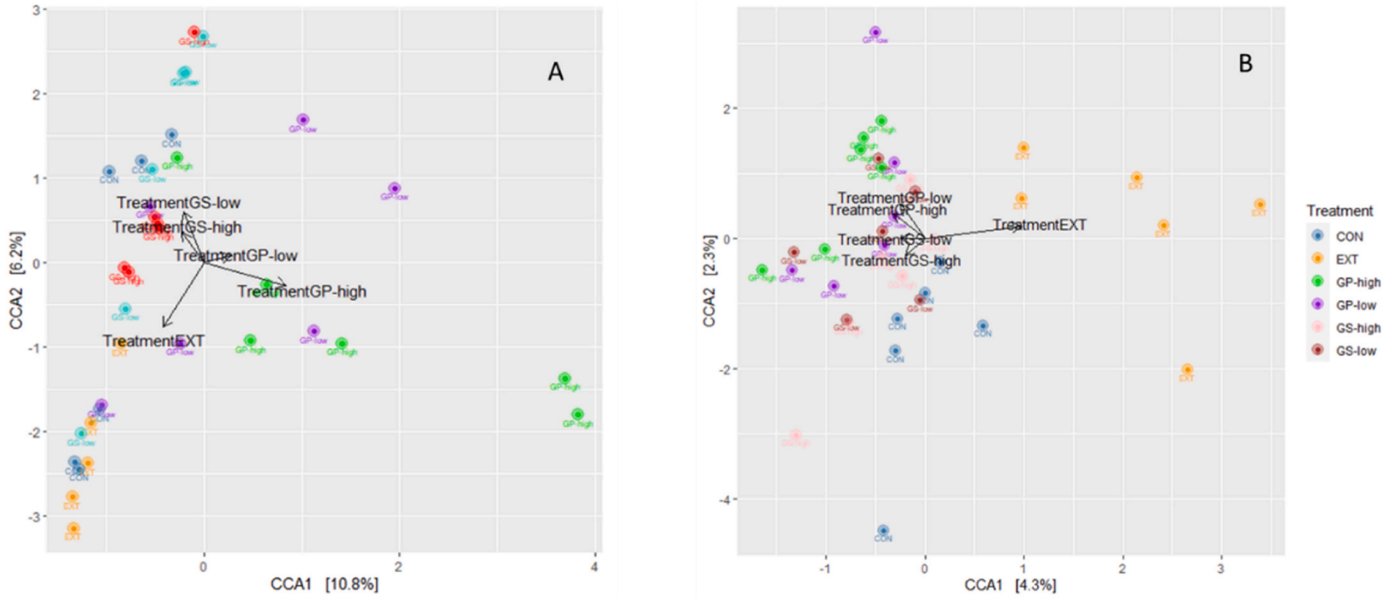

Fig. 2. Canonical Correspondence Analysis (CCA) plots for fungal (A) and bacterial and archaeal (B) composition among treatments (diets including hay and concentrate mix without (CON) or with different inclusion levels of grape pomace (GP: GP-low = 10 %, GP-high = 20 %), grapeseed meal (GS: GS-low = 5 %, GS-high = 10 %) or top-dressed with grapeseed extract at a 3.4 % of diet dry matter (EXT).

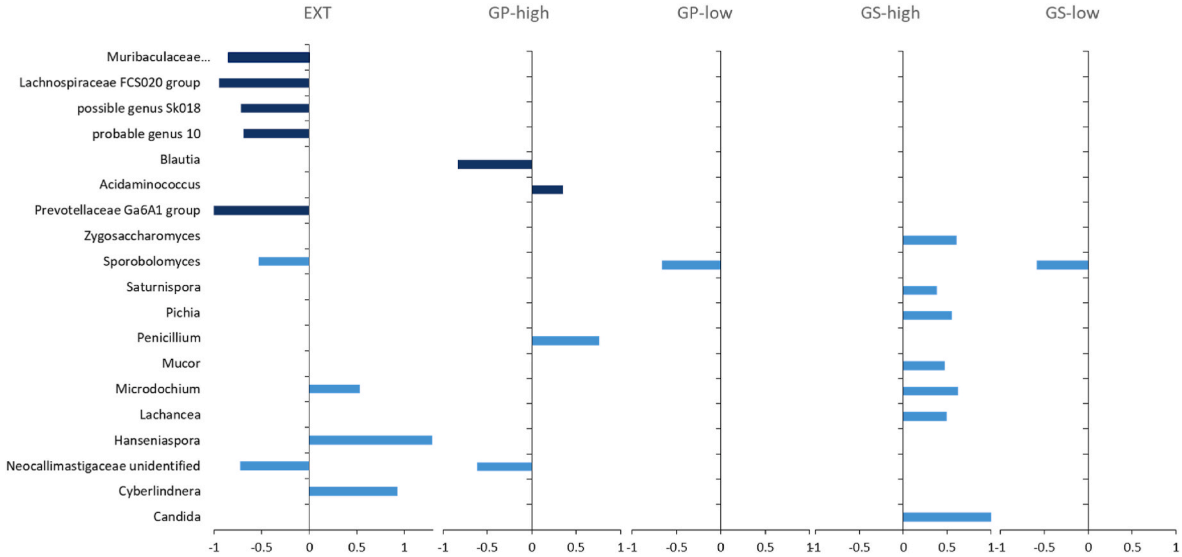

Fig. 3. Changes in fungi (light blue bars) and bacteria (dark blue bars) at the genus level following the inclusion of grape pomace (GP: GP-low = 10 %, GP-high = 20 %), grapeseed meal (GS: GS-low = 5 %, GS-high = 10 %) or top-dressed with grapeseed extract at a 3.4 % of diet dry matter (EXT). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

abundance but were exclusively presented in EXT and WBP samples (Table 2). Three species of *Pichia* (*P. sporocuriosa*, *P. kluyveri, P. terricola*) were elevated by GS-high, of which *P. sporocuriosa* showed the strongest increase (Fig. 4). Other species increased by GS-high were *Hanseniaspora guilliermondii, Candida stellata, Lachancea thermotolerans, Mucor circinelloides, Saturnispora diversa, Zygosaccharomyces bailii* ($Q < 0.05$), and *Kazachstania exigua* ($Q = 0.064$). EXT increased read abundances of *Hanseniaspora valbyensis* ($Q < 0.05$) (Fig. 4), *Leucosporidium fragarium and Itersonilia pannonica* ($Q < 0.10$) compared to CON. GS-low increased *Zygosaccharomyces bailii* ($Q < 0.10$) while *Sporobolomyces roseus* was decreased in GS-low and GP-low compared to CON ($Q < 0.10$).

The composition of bacteria differed among treatments at the taxonomy below the family level. The main changes were a negative effect of EXT on *Prevotellaceae* Ga6A1 group, probable genus 10, possible genus Sk018, *Lachnospiraceae* FCS020 group ($Q < 0.05$, Fig. 3), and to a lesser extent F082 uncultured rumen bacterium ($Q = 0.09$, data not shown). Other changes were decreased *Blautia* spp. and increased *Acidaminococcus* spp. by GP-high ($Q < 0.05$). Affected genera *Acidaminococcus* spp., *Muribaculaceae* uncultured bacterium, and possible genus Sk018 were absent or were relatively minor (<0.1 %) in the population of donor cow solids and the first two genera proliferated *in vitro* (Supplementary Table 1). The other five affected genera were more abundant in the donor cow samples (0.32 ± 0.15 % - 1.43 ± 0.30 %, mean ASV ± SD).

Treatments led to changes in read abundances of 16 bacterial species ($Q < 0.05$, Supplementary Fig. 2). Eight species showed a tendency compared to CON ($0.5 < Q \leq 0.10$). Selected species are presented in Fig. 5 representing different effects of different treatments. Major

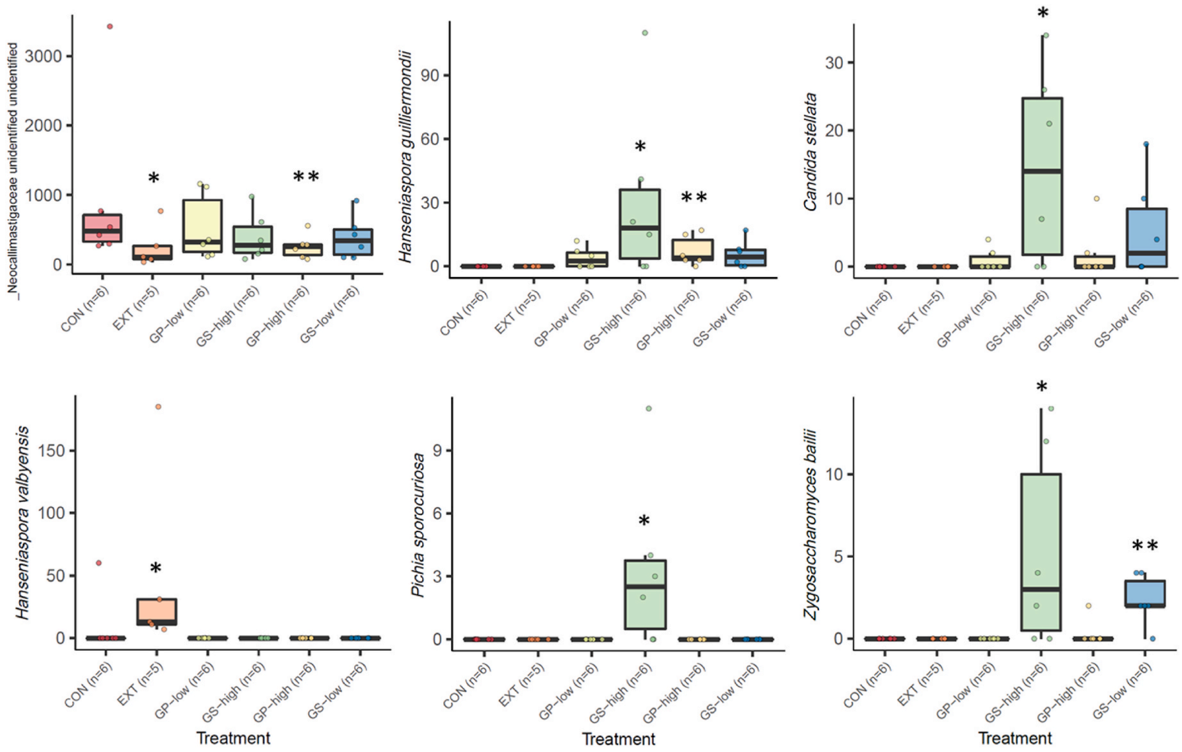

Fig. 4. Read abundance (amplicon sequence variants number) of some identified fungal species affected by treatment. FDR is a P value corrected for the false discovery rate. Treatments consisted of diets including hay and concentrate mix without (CON) or with different inclusion levels of grape pomace (GP: GP-low = 10 %, GP-high = 20 %), grapeseed meal (GS: GS-low = 5 %, GS-high = 10 %) or top-dressed with grapeseed extract at a 3.4 % of diet dry matter (EXT). *, **Asterisks indicate differences from CON at FDR <0.05 and 0.05 ≤ FDR ≤0.10, respectively). Each group n = 6, except EXT n = 5.

species like *Prevotellaceae* Ga6A1 group uncultured bacterium, *Treponema bryantii*, and *Butyrivibrio fibrisolvens* and the less abundant *Lachnospiraceae* UCG 009 uncultured rumen bacterium were decreased in EXT, but not in WBP treatments, compared to CON, while *Lachnospiraceae* UCG-009 uncultured bacterium was increased in EXT ($Q < 0.05$). *Treponema berlinense* was promoted by EXT and WBP treatments, especially GP groups. GS-high and GP-high increased unidentified rumen bacterium RFN19 and only GP-high increased *Acidaminococcus* uncultured bacterium compared to CON ($Q < 0.05$).

3.4. Correlations between microbiota and ruminal degradation

Correlations ($r > 0.6$ or $r < -0.6$ and a P-value <0.05) were detected for multiple bacterial genera and two fungal genera, the latter included *Saccaromyces* spp. and *Zymoseptoria* spp. that were negatively correlated to degradation of fiber (i.e., neutral detergent fiber) (Table 3). Some bacterial genera showed frequent correlations with the degradation of different nutrients. For instance, *Saccharofermentans* spp. was positively correlated with the degradation of DM, organic matter, crude protein, and fiber. *Succinivibrio* spp. was negatively correlated with degradation of DM, crude protein, and fiber, but was positively correlated to ammonia production (mmol/g degraded crude protein). Uncultured *Erysipelotrichaceae* bacterium showed the highest correlation ($r = 0.724$) with neutral detergent fiber degradation. *Succinivibrionaceae* UCG-001 showed the highest correlation with CH_4 production (ml/g degraded organic matter, $r = 0.782$), followed by *Bacteroidales* bacterium 22 (0.712) and *Methanimicrococcus* spp. (0.689). *Succinivibrionaceae* UCG-001 also showed the strongest positive correlation with ammonia production ($r = 0.752$), while *Lachnospiraceae* UCG-009 and *Eubacterium xylanophilum* group were negatively correlated ($r = -0.650$). Some of the genera affected by treatments (Fig. 2) also correlated with nutrient degradation ($P \leq 0.05$) but with weaker coefficients than the cutoff threshold (data not shown). Accordingly, the correlation coefficients between the degradation of neutral detergent fiber and bacterial genera were as follows: *Blautia* spp. ($r = 0.36$), possible genus Sk018 ($r = 0.41$), probable genus 10 ($r = 0.35$), *Acidaminococcus* spp. ($r = -0.60$), and *Muribaculaceae* uncultured bacterium ($r = -0.45$). The correlation coefficients between the degradation of crude protein and bacterial genera were as follows *Lachnospiraceae* FCS020 group ($r = 0.54$), possible genus Sk018 ($r = 0.40$), probable genus 10 ($r = 0.52$), *Acidaminococcus* spp. ($r = -0.33$), and *Prevotellaceae* Ga6A1 group ($r = 0.35$).

4. Discussion

4.1. Bacteria and archaea

Previously, we showed that the many bacteria in the liquid fraction were suppressed by EXT but less so by other WBP treatments especially at low dosages [10]. Still, the different bacterial compositions resulted in similar beneficial effects of EXT and other WBP on reducing protein degradation and nitrogen metabolism in the rumen. In the present study, a negative effect of EXT was also detected on bacteria in the solid fraction, albeit for fewer genera as compared to those in the liquid fraction. This may suggest more tolerance of solid rumen microbiota to grape phenols. However, the common rumen bacterial species *Butyrivibrio fibrisolvens* and *Treponema bryantii* appeared to be sensitive to rapid exposure to grape phenols delivered in an extract form. We found that both species were exclusively suppressed by EXT and this occurred persistently in both solid (Fig. 5) and liquid fractions [10]. As shown by electron microscopy, *T. bryantii* is associated with plant cell walls [34], while *B. fibrisolvens* is not [35]. Both species are non-cellulolytic but were reported to be involved in fiber digestion due to their interaction with other cellulolytic bacteria [34,36,37]. In support of this notion, we found multiple folds more read abundances of both species in solids than in liquid fractions. The richness *B. fibrisolvens* in the solid fraction underlines its associations in close proximity with other fiber-adherent

Table 2
Amplicon sequence variants numbers (mean ± SD) of identified fungal species detected in all samples, or exclusively and mostly in the solid inocula from the donor cows or RUSITEC samples.

Species/Sample	Donor cows (n = 6)	RUSITEC[a]						% Positive COW only	% Positive CON only	% Positive EXT only	% Positive GS + GP only
		CON (n = 6)	EXT (n = 5)	GP-high (n = 6)	GP-low (n = 6)	GS-high (n = 6)	GS-low (n = 6)				
unassigned Orpinomyces	2795 ± 1029	1701 ± 1657	967 ± 1376	3210 ± 3537	4343 ± 3322	4218 ± 2853	6251 ± 4766	100	100	100	100
unassigned Neocallimastigaceae*	1189 ± 810	955 ± 1226	251 ± 303	256 ± 172	530 ± 480	396 ± 341	388 ± 312	100	100	100	100
Cyllamyces aberensis	519 ± 274	68 ± 141	140 ± 210	116 ± 130	81 ± 71	130 ± 157	93 ± 98	100	100	100	96
Caecomyces communis	240 ± 206	0	0	0	0	0	0	100	0	0	0
unidentified *Piromyces*	228 ± 174	0	0	0	0	0	0	100	0	0	0
*Sporobolomyces roseus**	0 ± 1	1 ± 1	0	1 ± 1	0	1 ± 2	0	17	50	0	17
Saccharomyces cerevisiae	0 ± 1	1272 ± 1188	1177 ± 1225	4402 ± 4915	2618 ± 3232	1967 ± 3021	1057 ± 1584	17	100	100	100
Cystofilobasidium macerans	0	9 ± 8	24 ± 50	11 ± 20	6 ± 7	26 ± 40	9 ± 12	0	83	60	63
Holtermanniella takashimae	0	11 ± 19	14 ± 15	18 ± 25	5 ± 5	31 ± 36	10 ± 14	0	83	80	63
Vishniacozyma victoriae	0 ± 1	71 ± 80	40 ± 27	82 ± 68	69 ± 86	109 ± 108	45 ± 43	17	100	100	100
Wallemia muriae	0	15 ± 8	36 ± 32	16 ± 18	20 ± 21	11 ± 9	16 ± 11	0	83	80	88
Leucosporidium golubevii	1 ± 1	3 ± 3	9 ± 14	7 ± 7	1 ± 2	6 ± 6	3 ± 5	17	67	60	58
Vishniacozyma psychrotolerans	0	4 ± 6	1 ± 1	4 ± 4	2 ± 2	5 ± 5	6 ± 10	0	83	40	50
Aspergillus glaucus	0	4 ± 4	3 ± 3	2 ± 3	3 ± 3	4 ± 2	7 ± 10	0	67	60	71
unassigned Erythrobasidiales	0 ± 1	4 ± 5	2 ± 2	4 ± 3	2 ± 3	4 ± 2	5 ± 6	17	67	60	71
Scopulariopsis candida	0	5 ± 6	9 ± 16	3 ± 6	8 ± 10	6 ± 8	3 ± 3	0	50	40	58
Symmetrospora coprosmae	0	2 ± 3	4 ± 2	1 ± 1	1 ± 2	2 ± 2	2 ± 2	0	50	80	46
unassigned Mrakiella	0	1 ± 1	3 ± 4	1 ± 2	23 ± 53	1 ± 1	11 ± 25	0	67	40	38
*Cyberlindnera rhodanensis**	0	15 ± 37	107 ± 200	0	0	0	0	0	17	100	0
*Hanseniaspora valbyensis**	0	10 ± 24	49 ± 76	0	0	0	0	0	17	60	8
*Leucosporidium fragarium**	0	0 ± 1	2 ± 2	2 ± 3	0	0	0	0	17	100	0
*Itersonilia pannonica**	0	0	1 ± 2	0 ± 1	0	1 ± 2	0	0	0	40	8
*Candida stellata**	0	0	0	2 ± 4	1 ± 2	15 ± 14	5 ± 7	0	0	0	46
*Hanseniaspora guilliermondii**	0	0	0	7 ± 7	4 ± 5	31 ± 42	6 ± 7	0	0	0	67
*Zygosaccharomyces bailii**	0	0	0	0	0	5 ± 5	2 ± 2	0	0	0	42
*Kazachstania_exigua**	0	0	0	2 ± 2	1 ± 2	0	0	0	0	0	21
*Lachancea thermotolerans**	0	0	0	0	0	5 ± 6	1 ± 1	0	0	0	21
*Pichia sporocuriosa**	0	0	0	0	0	3 ± 4	0	0	0	0	17
*Pichia terricola**	0	0	0	0	0	3 ± 4	0 ± 1	0	0	0	17
*Pichia kluyveri**	0	0	0	0	0	3 ± 4	1 ± 2	0	0	0	17
*Saturnispora diversa**	0	0	0	0	0	4 ± 4	1 ± 1	0	0	0	17
*Mucor circinelloides**	223 ± 379	0	0	0	0 ± 1	5 ± 5	0	33	0	0	17

*Treatment(s) differed from CON ($P < 0.05$ or $0.05 < P < 0.10$).

[a] Treatments consisted of diets including hay and concentrate mix without (CON) or with different inclusion levels of grape pomace (GP: GP-low = 10 %, GP-high = 20 %), grapeseed meal (GS: GS-low = 5 %, GS-high = 10 %) or top-dressed with grapeseed extract at a 3.4 % of diet dry matter (EXT).

microbiota. Given the importance of these rumen commensal bacteria in fiber degradation, using grapeseed meal and pomace seems preferable to using grapeseed extract in stabilizing the rumen bacterial community while still harnessing the beneficial effect of grape phenols.

Ten members of *Spirochaetes* were identified as *Treponema* spp., among these only *T. bryantii* and *T. berlinense* were affected by treatment. In contrast to *T. bryantii*, *T. berlinense* showed lower abundance in general, being almost absent in CON samples. However, it was detected in all WBP treatments and was enriched by GP treatments (Fig. 5). At first glance, this may hint at a transfer of WBP-associated spirochaetes, however, this species was already present in the donor cow solids (3–19 ASVs number, detailed data not shown). Therefore, the results rather suggest the ability of WBP, especially GP, to maintain this species in *in vitro* conditions. Our findings underline the need for considering microbial communities in donor animals when discussing the treatment effects. The reason for better sustained *T. berlinense* by WBP diets is unknown but might be related to the presence of more lignified fiber contents as well as the favor of this species towards plant bioactive compounds. *T. berlinense* was originally described by Nordhoff et al. [38] and was an isolate of pig feces. To our best knowledge, fermentation functions of *T. berlinense* have not been reported but studies in humans have shown that this species appears to be enriched in traditional rural populations. As compared to those of urban Saudis, *T. berlinense* and *T. succinifaciens* were only present in the gut microbiome of rural Bedouins who regularly consume vegetables, fruit, and homemade fermented dairy products [39]. Likewise, a recent study showed that *Troponema* spp. was enriched in the gut of a traditional rural population with *T. berlinense* being commonly found in Amazonians [40]. Cross-transmission of *Spirochaetes* spp. between humans and animals and increased antibiotic use by urban populations were explanations given by the authors.

In our previous investigation, we revealed that, besides lowering

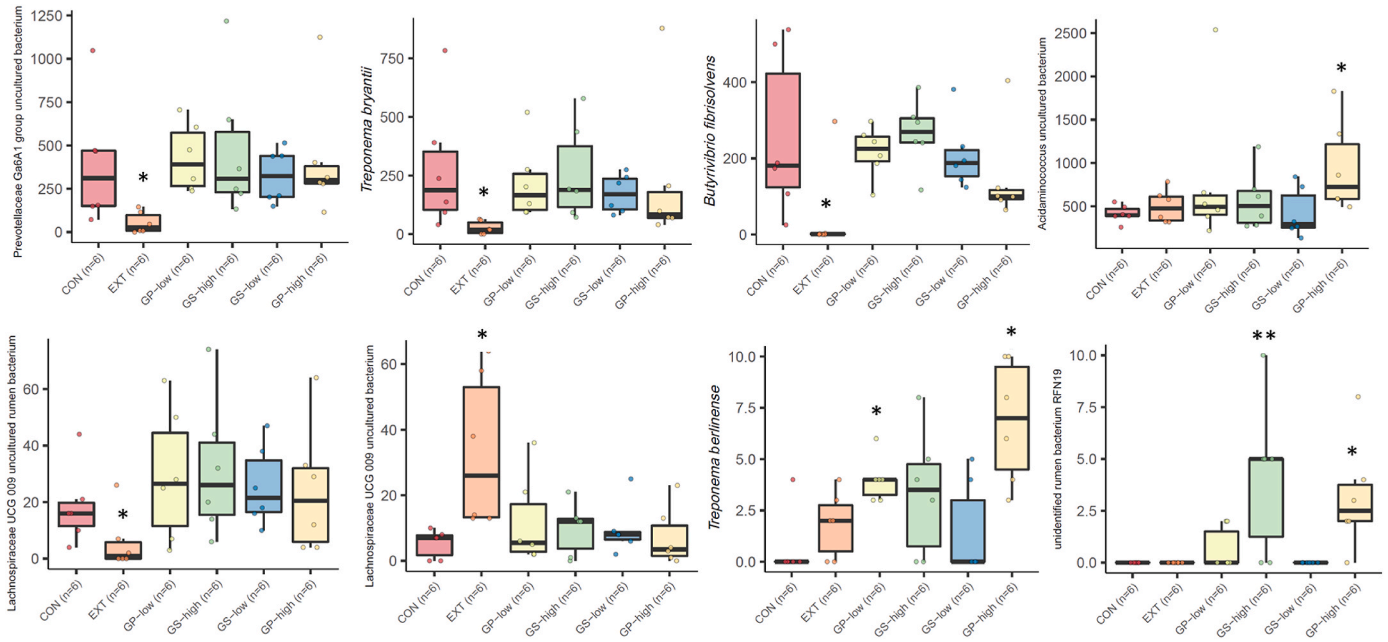

Fig. 5. Read abundance (amplicon sequence variants number) of some identified bacterial species affected by treatment. FDR is a P value corrected for the false discovery rate. Treatments (each n = 6) consisted of diets including hay and concentrate mix without (CON) or with different inclusion levels of grape pomace (GP: GP-low = 10 %, GP-high = 20 %), grapeseed meal (GS: GS-low = 5 %, GS-high = 10 %) or top-dressed with grapeseed extract at a 3.4 % of diet dry matter (EXT). *’ **Asterisks indicate differences from CON at FDR <0.05 and 0.05 ≤ FDR ≤0.10, respectively).

ammonia production by WBP treatments, nutrient degradation was also affected depending on treatments. Specifically, EXT decreased the degradation of crude protein, while GP-low and GP–high decreased fiber degradation [10]. In that study, we found very few associations between the liquid microbiota with the nutrient degradation and fermentation efficiency variables. Conversely, in the present study, in the solid population, several bacterial genera showed correlations with nutrient degradation variables (Table 3). Many rumen bacteria have proteolytic activity via cell-bound proteases after their attachment to feed particles [41]. Correlations of solid bacteria with protein degradation in the present work reflected this. Also, four out of the five genera exclusively suppressed by EXT (*Lachnospiraceae* FCS020 group, possible genus Sk018, probable genus 10, and *Prevotellaceae* Ga6A1 group) were positively correlated with crude protein degradation, though their correlations were weaker (r = 0.35–0.54) than the genera listed in Table 3. Rumen microbes, especially cellulose-digesting bacteria, also utilize branched-chain short-chain fatty acids that are products of microbial protein degradation [42]. Members of *Prevotellaceae* and probable genus 10 seem to be stronger utilizers of branched-chain short-chain fatty acids [43]. Thus, decreased abundances of bacteria by EXT reflected the disturbance of this complex relationship among the rumen microbes, in addition to the effect of polyphenols binding soluble dietary protein, thereby protecting the protein from microbial attack [44]. However, the results of solid microbiota cannot be linked to the effect of GP on decreasing fiber degradation. Since grape pomace contains stems that are woody components of vine bagasse [45], the physical property of the woody components (i.e. lignified fiber) in the GP was likely the main reason for reduced fiber degradation.

Among the bacterial and archaeal genera in the solid fraction showing considerable correlations with the nutrient degradation and fermentation efficiency variables (Table 3), standing out were *Saccharofermentans* spp. and *Succinivibrio* spp., which showed persistent positive and negative correlations, respectively, with the degradation of neutral detergent fiber, protein, and dry matter. They were common genera in the donor cows as well (data not shown). Their opposite correlations might display their competition for substrates (e.g., glucose) as well as attribution to the high-fiber diets used in the present study, especially with more GP levels. Both *Sacchrofermentans* spp. and *Succinivibrio* spp. ferment glucose to succinic acid, acetic acid, and formate [46,47]. *Saccharofermentans* spp. seem to be involved in fiber degradation as it was enriched in cows recovered from subacute ruminal acidosis after transplantation of rumen contents from healthy donor cows fed a high forage diet [48]. On the contrary, the propionate-producing *Succinivibrio* spp. proliferates in animals fed high grain diets, and is, therefore, considered a fermenter of hydrolytic products of starch [46].

4.2. Fungi

We showed here that including WBP shaped the fungal composition already at the phylum level. Although Neocallimastigomycota was still a highly abundant phylum, which was in line with other reports in ruminants [49], the fungal composition shifted to more proportions of Ascomycota with GP-high and Mucoromycota with GS-high. When evaluating at lower taxonomical levels, it became apparent that GS-high was the most potent treatment that frequently increased the abundances of several genera of Ascomycobiota (*Candida* spp., *Lachancea* spp., *Microdochium* spp., *Pichia* spp., *Saturnispora* spp., and *Zygosaccharomyces* spp.) as well as the genus *Mucor* spp. of Mucoromycota (Fig. 3). Species elevated by GS-high included those naturally found on grapes (e.g., *Candida stellata*, *Hanseniaspora guilliermondii*, *Pichia kluyveri*, and *Lachancea thermotolerans*) [50,51], spoilage yeasts (e.g., *Zygosaccharomyces bailiias*) [52] as well as *Mucor circinelloides*, which is an environmental mold found in soil and decaying materials but can lead to infection [53]. *Hanseniaspora* was specifically elevated by EXT. These listed species were exclusively present in WBP treatments, but not in CON and donor cow solids (Table 2), which firmly underlines the introduction of these fungi into the rumen solid ecosystem by WBP. These yeasts ferment sugars, and some species, such as *Candida stellata* and *Lachancea thermotolerans*, can tolerate up to 10 % ethanol [54,55] and so are more persistent in fermentation. Notably, GP groups having more inclusion rates than GS-high did not result in stronger changes, suggesting concentrated loads of grape and wine yeasts in the seed products.

Table 3
Correlations[a] between microbial genera and nutrient digestibility variables.

Variable	Genus[b]	*r*
Fiber[c] degradation (% of supply)	*Coprococcus* 2	0.659
	Eubacterium ruminantium group	0.705
	Lachnospiraceae uncultered	0.631
	Pseudobutyrivibrio	0.632
	Ruminococcus 1	0.674
	Saccharofermentans	0.715
	Saccharomyces	−0.630
	Succinivibrio	−0.680
	uncultured *Erysipelotrichaceae* bacterium	0.724
	Zymoseptoria	−0.622
Methane production (ml/g degraded OM)	*Bacteroidales* bacterium Bact_22	0.712
	Bacteroidales BS11 gut group uncultured bacteria	0.607
	Lachnospiraceae XPB1014 group	−0.619
	Methanimicrococcus	0.635
	Ruminococcus 2	0.689
	Succinivibrionaceae UCG-001	0.782
	Prevotellaceae Ga6A1 group	−0.639
Crude protein degradation (% of supply)	*Eubacterium coprostanoligenes* group	0.611
	Eubacterium ruminantium group	0.631
	Lachnospiraceae NK4A136 group	0.613
	Lachnospiraceae UCG-009	0.609
	Saccharofermentans	0.619
	Succinivibrio	−0.619
Ammonia concentration (mmol/g degraded crude protein)	*Bacteroidales* bacterium Bact_22	0.631
	Eubacterium xylanophilum group	−0.648
	Hyphopichia	0.614
	Lachnospiraceae UCG-009	−0.655
	Methanimicrococcus	0.607
	Solobacterium	0.604
	Succinivibrio	0.618
	Succinivibrionaceae UCG-001	0.752
Dry matter degradation (% of supply)	*Saccharofermentans*	0.624
	Succinivibrio	−0.607
Organic matter degradation (% of supply)	*Saccharofermentans*	0.651
Ash degradation (% of supply)	hoa5-07d05 gut group	−0.649
Short-chain fatty acids (mmol/g degraded organic matter)	*Ruminococcus gauvreauii* group	−0.606

[a] Only genera with correlation coefficients of 0.600 and above or −0.600 and below are listed.

[b] Fungal genera are presented in a bold font.

[c] Neutral detergent fiber representing the total content of cellulose, hemicellulose, and lignin.

It must be acknowledged that the genomic data cannot indicate if the detected species were viable. Notably, unlike *Saccharomyces cerevisiae*, the non-*Saccharomyces* yeasts were present in very small abundances in RUSITEC samples, even when elevated by treatment. Many of these taxa are oxidative-fermentative yeasts [55]. Based on these grounds, the sequenced mycobiota represented only remnants of DNA of WBP microbes, or they were viable but could not compete with the rumen commensal populations. Either way, these data suggest limited microbial hazard of using WBP up to 20 % in cattle rations. Still, batch-to-batch variation should not be overlooked. Many factors can determine the composition and loads of microorganisms on grapes, in wine, fermented wine must, and consequently in the by-products. The composition of non-*Saccharomyces* yeasts of grape berries varies depending on the geographical origin of the grapes [56]. In our RUSITEC samples, the sequenced data indicated the presence of *Aspergillus* spp., *Alternaria* spp., and *Penicillium* spp. albeit in low abundance. For *Penicillium* spp., it was more prevalent in GP-high (Supplementary Fig. 3). Members of these fungal genera produce mycotoxins [57]. The aspect of chemical hazards (e.g., mycotoxins and pesticides) needs to be addressed in the future. These harmful compounds are already an existing concern regarding wine safety [58].

Anaerobic fungi produce high levels of cellulases, hemicellulases, and xylanases and therefore they actively degrade plant cell walls [59]. They are also better at colonizing and degrading lignified tissues than bacteria are [59]. Different from our expectation, we did not see strong increases in common rumen fungi like Neocallimastigomycota in response to the inclusion of GS or GP. We also did not observe correlations of the commensal rumen fungi with fiber degradation either. On the contrary, EXT and GP-high decreased members of Neocallimastigomycota. This reflects two things. First, like bacteria, some commensal rumen fungi were likely sensitive to high levels (>3 %) of grape phenols. However, the shaped community was still able to carry out the fiber-degrading activity. Second, the decreased fiber degradation by GP was mainly due to the woodier components in GP.

It must be noted that like what was observed in bacteria, not all rumen fungi in the cow rumen can flourish under *in vitro* conditions. Here, *Piromyces* spp. and *Caecomyces* spp. were not able to be maintained in the RUSITEC system after 10 days of incubation, while several other taxa absent in the donor cow solids were present in RUSITEC samples including CON (Table 2). These minor taxa may have come with the ingredients used in diets and were not further affected by WBP. The absolute similarities between donor cow solids and *in vitro* samples are not to be expected because of different matrixes (diets) and missing host and barn factors that cannot be simulated *in vitro* [17]. Nevertheless, compared to static batch cultures, semi-continuous RUSITEC systems can maintain a more natural microbiome composition over time [60] and thus represent the best *in vitro* tool for studying rumen microbiota and activity thus far. Our data further underline that it is also crucial to study the community of the native inoculum and the selection force of your test *in vitro* condition on the established community when interpreting the effect of a treatment on the microbiota data.

5. Conclusions

Our data suggests that when delivered in the form of extract, grape phenols could possess a negative effect on rumen commensal bacteria and fungi in the solid fraction. The inclusion of WBP, especially grapeseed, led to the introduction of several yeasts associated with grapes and wine in the rumen community, but their viability cannot be confirmed with the genomic data. However, their low abundances suggest that potential microbial hazards to animals might be limited when using WBP up to 20 % in cattle rations. However, this can only be confirmed by *in vivo* data. Members of the solid bacteria and fungi affected by the inclusion of WBP were not the strongest contributors to the *in vitro* ruminal degradation of nutrients observed in this study. Altogether, grape pomace and grape seed meal could be considered as functional feed for cattle. However, the microbial safety of feeding these by-products needs vigilance. Furthermore, we underline the necessity of considering donor cow microbiota when interpreting the test factors in *in vitro* fermentation.

Ethical statements

Donor cows were kept and handled according to the Austrian guidelines for animal welfare. Use of these cannulated cows does not require ethical clearance.

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CRediT authorship contribution statement

Ratchaneewan Khiaosa-ard: Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Cátia Pacífico:** Writing – review & editing, Writing –

original draft, Visualization, Software, Formal analysis, Data curation. **Mubarik Mahmood:** Writing – review & editing, Investigation. **Elsayed Mickdam:** Writing – review & editing, Investigation. **Julia Meixner:** Writing – review & editing, Investigation. **Laura-Sophie Traintinger:** Writing – review & editing, Investigation. **Qendrim Zebeli:** Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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