Application of multi-omics to investigate the effect of Pinus koraiensis cone essential oil on rumen methane emission, microbial community, and metabolites

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Abstract

Background

Enteric methane (CH_4) excreted by ruminants is a major source of anthropogenic greenhouse gas emissions in the global environment. *Pinus koraiensis* cone essential oil (PEO) contains functional compounds such as monoterpene hydrocarbons, which can directly affect the microbiota and their function in the rumen. Previously, we found that PEO oral administration during the growing phases of goats reduced CH_4 emissions and was associated with the rumen prokaryotic microbiota. However, a more comprehensive analysis of the rumen microbiota and metabolites are needed. The objective was to elucidate the potential microbial features that underpin CH_4 mitigation in goats using metataxonomics (prokaryotes, protozoa, and fungi) and metabolomics (rumen fluid and serum). Ten fattening Korean native goats were divided into two dietary groups: control (CON; basal diet without additives) and PEO (basal diet + 1.5 g/d of PEO), using a 2 × 2 crossover design for 11 weeks. Methane measurements were conducted every four consecutive days for 24–27 d.

Results

Oral administration of PEO reduced CH_4 concentrations in the exhaled gas from eructation by 12.0–13.6% ($P < 0.05$). Although the microbiota structure, including prokaryotes, protozoa, and fungi, was not altered after PEO administration, MaAsLin2 analysis revealed that *Selenomonas*, Christensenellaceae R-7, and *Anaerovibrio* were enriched in the PEO group ($Q < 0.1$). Co-occurrence network analysis revealed that the Bacteroidales RF16 group and *Anaerovibrio* were the keystone genera in the CON and PEO groups, respectively, with fungal genera exclusively found in the PEO group but not identified as keystone taxa. Predicted function analysis using CowPI, CH_4 metabolism was enriched in the CON group, whereas metabolism of sulfur ($P < 0.001$) and propionate ($P < 0.1$) were enriched in the PEO group. Random forest analysis identified eight ruminal metabolites, including propionate, that were altered after PEO administration, with predictive accuracy ranging from 0.75 to 0.88. *Selenomonas* was positively correlated with propionate and co-occurred with it.

Conclusions

The results provide an understanding of how PEO oral administration affects the ruminal microbial community and its functions in the rumen, as well as its linkages with rumen metabolites and host health, ultimately leading to the reduced CH_4 emissions.

Background

Ruminants are unique in that they harbor a mutualistic microbial community in their rumen that converts complex fibrous feed into valuable commodities, such as milk and meat, suitable for human consumption [1, 2]. Therefore, the rearing of ruminant livestock is crucial for addressing global food security challenges and reducing poverty, especially in a world with a rapidly growing population [3].

However, a major challenge associated with ruminants is their significant contribution (9.5 %) to athropogenic greenhouse gas emissions [4], which has a negative impact on the environment. Moreover, the energy lost as CH_4 from ruminants ranges between 2 and 12% of gross energy intake [5]. Therefore, it is important to find ways to mitigate these emissions while maintaining benefits for the ruminant industry.

Numerous nutritional strategies have been used to reduce CH_4 emissions in ruminants, enhance feed efficiency, and minimize energy loss from diets. Recently, nitrooxy compound (3-nitrooxypropanol) and red seaweed (*Asparagopsis taxaformis*) have been found to be effective rumen CH_4 inhibitors [6, 7]. Both inhibitors suppress rumen methanogenesis and improve production efficiency in long-term experiments [8, 9]. Although the mechanism of action has been established [10, 11]. 3-nitrooxypropanol is yet to be approved by the Food and Drug Administration for commercial use in the United States [12], and the effect of 3-nitrooxypropanol on the composition and function of the rumen microbiome has not been comprehensively studied [13]. Additionally, Muizelaar et al. [14] reported that the bromoform from *A. taxiformis* could be excreted in urine and milk, causing abnormalities in the rumen wall papillae. Therefore, it is necessary to identify new natural sources that can reduce CH_4 production without detrimental effects on ruminal fermentation, thereby providing an effective and sustainable strategy for controlling CH_4 emissions.

Previously, we evaluated *Pinus koraiensis* cone essential oil (PEO) using a series of *in vitro* systems [15], showing it could reduce the CH_4 production up to 65%. Moreover, a previous *in vivo* study revealed that oral administration of PEO lowered CH_4 emissions by up to 16.5% and altered the composition of rumen prokaryotic bacteria without affecting dry matter intake during the growing phases of goats [16]. In particular, pyruvate oxidation, an aspect of carbohydrate metabolism, was enriched after PEO administration, which enhanced the molar proportions of acetate and butyrate. Furthermore, the rumen microbiota related to the production of these metabolites were also enriched.

In light of these findings, the present study aimed to investigate the potential of PEO to reduce enteric CH_4 emissions and its effects on multi-kingdom rumen microbiota prokaryotes (bacteria and archaea), and eukaryotes (protozoa, and fungi). Moreover, we sought to compare changes related to rumen and serum metabolomes during the fattening phases of goats following oral administration of PEO. Investigation of rumen and serum metabolomes is crucial, as it can help identify CH_4 emission-related metabolism and host health status.

Through multi-omics analyses (metataxonomics and metabolomics), we expanded our knowledge of the interactions among the ruminal microbial community, rumen metabolome, and host metabolome.

Results

Growth performance, rumen fermentation parameters, and CH_4 emissions

Oral administration of PEO did not affect the final BW, BW changes, DM intake (DMI), or DM digestibility (DMD) (Table 2). The average pH and NH_3-N concentrations in ruminal fluid did not differ between the CON and PEO groups.

Table 2
DM intake, growth performance, and rumen fermentation characteristics for the dietary treatments

Item	Treatment		SEM	*P*-value
	CON	PEO		
Initial BW, kg	42.0	41.7	0.51	0.544
Final BW, kg	41.2	41.2	0.55	0.972
BW change[1], kg	-0.81	-0.47	0.29	0.275
DMI, kg/d	1.29	1.27	0.04	0.586
DMD, %	0.70	0.70	0.02	0.927
pH	7.09	7.00	0.07	0.268
NH_3-N, mg/dL	8.61	8.86	0.15	0.133

SEM: standard error of the mean; amplicon sequence variant; CON: without PEO; PEO: *Pinus koraiensis* essential oil; BW: body weight; DM: dry matter; DMI: dry matter intake; DMD: dry matter digestibility; NH_3-N: ammonia nitrogen.

[1]Over 28 d.

Methane concentrations in the gas exhaled from respiration (ppm-m, ppm/$BW^{0.75}$, and ppm/kg DMI) did not differ between the CON and PEO groups (Table 3). However, when CH_4 concentration was expressed as ppm/kg digestible DMI (DDMI), the CH_4 concentration tended to be lower (P = 0.092) in the PEO group. Furthermore, eructation (ppm-m, ppm/$BW^{0.75}$, and ppm/kg of DDMI) was significantly lower (P = 0.042, P = 0.044, and P = 0.036) in the PEO group compared to the CON group. Additionally, when CH_4 concentration was expressed as ppm/kg DMI, the CH_4 concentration during eructation tended to be lower (P = 0.079) in the PEO group than in the CON group. As a result, CH_4 emissions from eructation were lowered in the PEO group by approximately 12–13.6% compared to the CON group.

Table 3
Enteric methane emission for the dietary treatments

Item	Treatment		SEM	*P*-value
	CON	PEO		
CH_4 from respiration				
ppm-m	16.9	16.0	0.75	0.235
ppm/$BW^{0.75}$	0.55	0.52	0.03	0.266
ppm/kg of DMI	13.1	12.4	0.73	0.353
ppm/kg of DDMI	24.4	22.6	0.90	0.092
CH_4 from eructation				
ppm-m	61.2	53.6	3.15	0.042
ppm/$BW^{0.75}$	1.98	1.74	0.10	0.044
ppm/kg of DMI	47.6	41.9	2.82	0.079
ppm/kg of DDMI	88.3	76.3	4.80	0.036

SEM: standard error of the mean; CON: without PEO; PEO: *Pinus koraiensis* essential oil; CH_4: methane; BW: body weight; DMI: dry matter intake; DDMI: digestible dry matter intake.

Alpha and beta diversity and co-occurrence interaction

After denoising and quality filtration (Q-score > 20), an average of 73,306 ± 2,842 (mean ± standard error) sequences were obtained for prokaryotes. For protozoa, 51,079 ± 2,062 sequences were retained, whereas 55,194 ± 5,882 sequences were obtained for fungi. Based on the minimum number of sequences obtained for each kingdom across all samples, rarefaction and analyses were performed on 54,511, 34,579, and 9,570 sequences per sample for prokaryotes, protozoa, and fungi, respectively. Good's coverage index was 99.9% for all kingdom sequences, indicating that the sequencing depth adequately represented the rumen microbial community. The alpha diversity measurements for each rumen kingdom (Chao 1 estimates, Evenness, Shannon's and Simpson's indices) did not show any significant differences between the CON and PEO groups (Table 4). Furthermore, PCoA and PERMANOVA revealed that none of the beta-diversity metrics, such as weighted and unweighted UniFrac distances, exhibited any significant differences between the CON and PEO groups in relation to the rumen kingdoms (Fig. 1).

Table 4
Summary of alpha diversity measurements of the rumen microbiota for the dietary treatments

Item	Treatment		SEM	*P*-value
	CON	PEO		
Bacteria and archaea				
Chao1 estimate	628	533	82.1	0.283
Evenness	0.78	0.76	0.02	0.491
Shannon's index	7.18	6.75	0.37	0.284
Simpson's index	0.98	0.97	0.01	0.244
Protozoa				
Chao1 estimate	43	40	2.27	0.339
Evenness	0.65	0.64	0.03	0.939
Shannon's index	3.51	3.41	0.20	0.657
Simpson's index	0.84	0.83	0.02	0.674
Fungi				
Chao1 estimate	384	419	62.0	0.579
Evenness	0.56	0.57	0.05	0.907
Shannon's index	4.56	4.75	0.42	0.647
Simpson's index	0.88	0.88	0.04	0.842

SEM: standard error of the mean; ASV: amplicon sequence variant; PD: phylogenetic diversity; CON: without PEO; PEO: *Pinus koraiensis* essential oil

Taxonomic classification for each kingdom in the rumen

In total, 22 phyla, 72 families, and 110 genera were assigned to prokaryotes. This study focused on major classified taxa, which were defined as occupying over 0.1% of the average relative abundance in at least one of the groups. Therefore, 14 phyla, 38 families, and 58 genera were identified (Fig. 2A). In total, 12 bacterial phyla were identified in the rumen samples. For protozoal and fungal genera, 5 and 14 phyla were identified, respectively, which occupied over 0.1% of the average relative abundance (Fig. 2B and 2C).

The Venn diagram indicates the common and exclusive identification of genera within the rumen kingdom in the CON and PEO groups (Fig. 2D). Among the prokaryotes, 95 of the 110 genera were shared between the CON and PEO groups. All genera of protozoa were shared between the CON and PEO groups,

whereas 22 of the 61 genera were shared by fungi. The CON group showed a higher number of specific genera of prokaryotes and fungi than the PEO group.

The dominant phyla were the following three bacterial phyla: Bacteroidota (CON, 51.0%; PEO, 50.6%; FDR, 1.000), Verrucomicrobiota (CON, 21.4%; PEO, 13.3%; FDR = 0.249), and Firmicutes (CON, 14.9%; PEO, 16.2%; FDR = 0.664) **(Table S2)**. Two ruminal archaeal phyla were identified: Thermoplasmatota (CON, 0.29%; PEO, 0.16%; FDR = 0.196) and Euyarchaeota (CON, 0.05%; PEO, 0.15%; FDR = 0.282). A total of 38 bacterial families were identified, the three most dominant were the following: Prevotellaceae (CON, 23.4%; PEO, 30.3%; FDR = 0.076), WCHB1-41 (CON, 18.4%; PEO, 11.3%; FDR = 0.111), and Rikenellaceae (CON, 7.95%; PEO, 7.38%; FDR = 0.758) **(Table S3)**. In total, 58 bacterial genera were identified, the three most dominant genera being WCHB1-41 (CON, 18.4%; PEO, 11.3%; FDR = 0.193), *Prevotella* (CON, 15.3%; PEO, 21.6%; FDR = 0.451), and Bacteroidales RF16 group (CON, 7.92%; PEO, 5.38%; FDR = 0.290) **(Table S4)**.

MaASlin2 analysis identified differentially abundant microbial genera in prokaryotes and fungi between the CON and PEO **(**Fig. 2E**)**. For prokaryotes, UG Methanomethylophilaceae (Q = 0.079 and Coef = -0.5732) and Oscillospiraceae UCG-002 (Q = 0.043 and Coef = -0.4677) were enriched in the CON group compared to the PEO group, whereas *Selenomonas* (Q = 0.079 and Coef = 0.3262), the Christensenellaceae R-7 group (Q = 0.021 and Coef = 0.4702), and *Anaerovibrio* (Q = 0.003 and Coef = 0.3174) were enriched in the PEO group. For the fungi, *Neocllimastix* (Q = 0.001 and Coef = -1.1320) and *Piromyce*s (Q = 0.038 and Coef = -0.4509) were enriched in the CON group.

Co-occurrence analysis of rumen microbial kindom

Based on co-occurrence network analysis using CoDiNA, 19 and 86 significant interactions were identified among the 105 overall edges exclusively found in the ruminal microbiota of the CON and PEO groups, respectively (Fig. 3). Only six genus-level nodes (prokaryotes: 6) were found in the CON group, whereas the PEO group had 20 genus-level nodes (prokaryotes: 17 and fungi: 3) (**Table S5**). Protozoa and Archaea were not found in either group. In the CON group, Bacteroidales RF16 was identified as the keystone genus, co-occurring with six prokaryotic bacterial taxa: Lachnospiraceae ND3007 group, Lachnospiraceae AC2044 group, *Gastranaerophilales*, *Quinella*, *Fibrobacter*, and *Treponema*. Four other prokaryotic bacterial genera were also mutually exclusive: *Succiniclasticum*, *Prevotella*, *Ruminobacter*, and UCG-010. In the PEO group, *Anaerovibrio* was identified as the keystone genus, co-occurring with 15 prokaryotic bacterial taxa, including *Succiniclasticum*, *Prevotella*, *Selenomonas*, *Ruminobacter*, *Fibrobacter*, and the Christensenellaceae R-7 group. In contrast, *Anaerovibrio* was mutually exclusive to four other prokaryotic bacterial genera and one fungal genus, specifically *Victivallaceae*, Lachnospiraceae ND3007, *Gastranaerophilales*, vadinBE97, and *Neocallimastix*.

Predicted functions of rumen prokaryotic bacteria, protozoa, and fungi

Functional characteristics of rumen prokaryotic bacteria were predicted using CowPI, a specific functional inference tool for the rumen microbiome. Carbohydrate metabolism was higher (P = 0.063) in the PEO group compared to the CON group. The PEO group exhibited enrichment in ascorbate and aldarate metabolism (P = 0.022), galactose metabolism (P = 0.023), pentose and glucuronate interconversions (P = 0.008), and starch and sucrose metabolism (P = 0.019), whereas the CON group showed enrichment in the citrate cycle (TCA cycle) (P = 0.069), glycolysis/gluconeogenesis (P = 0.078), propionate metabolism (P = 0.059), and pyruvate metabolism (P = 0.017) (Fig. 4A). Compared to the PEO group, the CON group exhibited enrichment in two amino acid metabolism pathways and one lipid metabolism pathway. Furthermore, CH_4 metabolism (P = 0.010) and carbon fixation pathways in prokaryotes (P = 0.034) were enriched in the CON group, whereas sulfur metabolism (P = 0.003) was enriched in the PEO group. Additionally, minor metabolic pathways in the rumen were affected by oral PEO administration. Similarly, PICRUSt2 was used to predict the potential fungal enzyme functions (Fig. 4B). Most enzyme functions were significantly higher or tended to be higher in the PEO group than those in the CON group. Among these, four enzymes were found to be enriched: cinnamyl-alcohol dehydrogenase (LDA = 2.750, P = 0.019), NADPH-hemoprotein reductase (LDA = 2.708, P = 0.006), adenosylmethionine-8-amino-7-oxononanoate transaminase (LDA = 2.747, P = 0.026), and chitinase (LDA = 2.711, P = 0.026).

As for the protozoal microbiota, the absence of an appropriate database for detecting functional pathways precluded further analysis.

Rumen and serum metabolome analyses

We identified 181 metabolites in rumen fluid and 162 in serum, each present in at least 50% of the samples. Of these, 36 metabolites in rumen fluid and 18 in serum showed significant changes with PEO administration (P < 0.1), forming the core focus of our study due to their altered abundance in response to PEO administration.

The rumen fluid metabolites were classified into 11 groups (Fig. 5A). The PLS-DA score plots were clearly separated (component 1,10.6%; component 2,8.50%) from the total variation (Fig. 5B). Concentration shifts were evident in metabolite categories such as carbohydrates (Fig. 5C), propionate precursors (Fig. 5D), and other metabolic intermediates related to CH_4 and sulfur metabolism (Fig. 5E). The total VFA and molar proportions of individual VFAs are shown in Fig. 5F. There were 17 metabolite pathways with FDR < 0.1, which is the cut-off value for relevance associated with rumen fluid (Fig. 5G). Detailed information on P and FDR values of rumen fluid metabolites and pathways are shown in **Tables S6** and **S7**.

Serum metabolites were classified into eight groups (Fig. 6A). The PLS-DA score plots were clearly separated (component 1,10.6%; component 2,8.50%) from the total variation (Fig. 6B). Serum metabolite changes were noted in lipids (Fig. 6C) and amino acids (Fig. 6D). There were four metabolite pathways with FDR < 0.1, which is the cut-off value for relevance associated with serum (Fig. 6E). Serum metabolites and liver enzymes are shown in Fig. 6F. Of these, only two measurements were significantly

affected. For example, calcium levels were significantly higher ($P = 0.042$) in the PEO group than in the CON group, whereas inorganic phosphate levels were significantly higher ($P = 0.048$) in the CON group than in the PEO group. Detailed information on *P* and FDR values of rumen fluid metabolites and pathways are shown in **Tables S8** and **S9**.

Correlation analysis among differential rumen microbiota, metabolites, and animal performance.

Significant and strong Spearman's rank correlations ($|r| \geq 0.6$, $P < 0.05$) between some of the rumen and serum metabolites and several major genera were found **(**Fig. 7**)**. In the rumen fluid **(**Fig. 7A**)**, *Selenomonas* was positively correlated with lactose, N-acetylcysteine, and propionate. Moreover, *Anaerovibrio* was positively correlated with lactulose and negatively correlated with succinate levels. The Christensenellaceae R-7 group was positively correlated with erythritol and formate but negatively correlated with galactitol. Furthermore, unclassified fungi (fungal genus) were positively correlated with propionate levels. In the serum **(**Fig. 7B**)**, *Anaerovibrio* and *Desulfovibrio* were positively correlated with 2-phenylpropionate and 3-indoxylsulfate. Theophylline was positively correlated with *Anaerovibrio*, whereas galactarate was positively correlated with *Desulfovibrio*. *Selenomonas* was negatively correlated with 2-phenylpropionate, 3-indoxylsulfate, thymol, and galactarate and positively correlated with acetoin. *Methanobrevibacter* and UG Methanomethylophilaceae were positively correlated with sacrcosine and 3-indoxylsulfate, respectively. Regarding animal performance **(**Fig. 7C**)**, only *Selenomonas* was positively correlated with the molar proportion of propionate. *Anaerovibrio*, *Succinivibrio*, and Succinivibrionaceae UCG-002 were negatively correlated with most of the CH_4 emission parameters, whereas Prevotellaceae UCG-003, *Quinella*, Victivallaceae, WCHB1-41, *Methanobrevibacter*, and *Caecomyces* (fungal genus) were positively correlated. Furthermore, protozoal genera, such as *Dasytricha* and *Isotricha*, were positively correlated with NH_3-N and the molar proportion of others (e.g., valerate, isovalerate, and isobutyrate), respectively.

To identify the linkages between CowPI data and rumen microbiota, we conducted Spearman's rank correlation analysis **(Fig. S1)**, revealing associations with carbohydrate metabolism, CH_4 metabolism, and various other metabolic pathways.

Microbe-metabolite interaction patterns associated after PEO administration: Co-occurrence analysis

In total, 181 metabolites were identified in the rumen. Spearman's rank correlation analysis was used to select the metabolites that showed significant alterations after PEO administration. Based on the correlation analysis results, 33 rumen metabolites were associated with PEO administration ($P < 0.05$) and were used in the RF model for prediction. Eight metabolites, N-acetylglycine, O-acetylcholine, malate, 2-phenylpropionate, galactol, propionate, desaminotyrosine, and fumarate, were selected using the RF model, with a mean decrease in accuracy of more than two (Fig. 8A). The model we constructed had an AUC of 0.75 to 0.88, indicating a high level of accuracy in predicting outcomes after PEO administration. Multi-omics biplots depicting microbe-metabolite interactions in the CON and PEO groups are presented

in Fig. 8B and **C**, respectively. Additionally, heat maps were generated to visualize the inferred conditional probabilities (> 2) of specific metabolites, which revealed distinct interaction patterns between microbes and metabolites in the CON and PEO groups.

Discussion

Oral administration of PEO did not have a significant effect on growth performance during the fattening phase. Previous studies found that the administration of PEO lowered total VFA production and NH_3-N during the growing phases of goats [16]. Although both experiments showed that DMI and BW were not affected by PEO administration, we observed a noticeable alteration in the proportions of individual VFAs. Specifically, the proportion of propionate exhibited significantly higher shifts in the fattening phase, whereas in the growing phase, the proportions of acetate and butyrate showed significant changes compared to the CON group. We hypothesize that this discrepancy in VFA proportions might be attributed to rumen development. In the growing phase, the need for butyrate arises as it promotes the development of rumen epithelial tissue [17], strengthens its protective barrier function [18], and aids in feed digestion and absorption, thereby promoting growth. Conversely, the higher propionate proportion during the fattening phase likely correlates with reduced CH_4 emissions. Propionate formation competes with methanogenesis for metabolic hydrogen (H_2) in the rumen [19]. This is because CH_4 production (eructation), which did not differ during the growing phase, was significantly lower than that in the CON group during the fattening phase. These changes suggest that oral administration of PEO may have influenced the metabolic pathways and microbial populations responsible for VFA production in the rumen or the development of the rumen in goats during the growing or fattening phase.

Most of the absorbed ruminal propionate in ruminants is used for gluconeogenesis in the liver, with the remainder being transported to different tissues through the blood vessels [20]. In the present study, because ruminal production of propionate leads to glucose production through gluconeogenesis in the liver, it was expected that the glucose concentration would be lower in the CON group, which had a low molar proportion of propionate. However, blood glucose concentrations did not differ between the CON and PEO groups, despite propionate metabolism being enriched in the PEO group. Therefore, these results suggest that the CON group maintained the glucose concentration by regulating gluconeogenesis to compensate for the relatively lower proportion of propionate and increased energy loss due to CH_4 production. In contrast, Choi et al. [16], we found no significant differences between the CON and PEO groups regarding ALT/SGPT (a marker of liver health; [21]) and creatinine (a marker of kidney health; [22]). Serum metabolomics analysis revealed a correlation between 3-indoxylsulfate and various genera, indicating that this metabolite is primarily derived from the microbiota and linked to tryptophan metabolism. Wang et al. [23] suggested that high concentrations of 3-indoxylsulfate are associated with liver disorders in ruminants. Interestingly, the PEO group exhibited lower concentrations of 3-indoxylsulfate compared to the CON group (Fig. 6). However, the established threshold for defining a high concentration of 3-indoxylsulfate in ruminants has yet to be determined. Nevertheless, these metabolites could be influenced by the active constituents of PEO, warranting further research. These findings

suggest that PEO administration does not have a discernible effect on liver and kidney health during the fattening phase in goats. Additional investigations are necessary to explore the potential differences in the physiological responses of goats to PEO administration during different growing phases.

Administration of PEO did not induce any alterations in the alpha and beta diversity metrics of any of the three kingdoms. However, some ruminal prokaryotic bacteria, archaea, and fungi were differentially enriched in the CON and PEO groups. Our results revealed that PEO administration enhanced succinate-producing bacteria such as *Succinivibrio*, Succinivibrionaceae UCG-002, and *Ruminobacter*. Specifically, members of Succinivibrionaceae (CON: 4.30% vs. PEO: 9.95%) are negatively correlated with methanogens [24], while being positively associated with high feed efficiency [25] and lower CH_4 production [26]. Moreover, PEO increased the abundance of *Prevotella* and *Selenomonas*. These bacteria utilize pathways for propionate production and consume H_2 via the succinate pathway through the fermentation of sugars and lactate [27]. It is likely that these pathways are the primary routes of H_2 consumption, which accumulates as a consequence of reduced methanogenesis. Furthermore, CH_4 emission parameters exhibited a strong negative correlation with succinate and propionate producers, such as *Anaerovibrio*, *Succinivibrio*, and Succinivibrionaceae UCG-002. These findings align with the enrichment of propionate metabolism in the PEO group, where these three genera were also enhanced. As a result, oral administration of PEO can enhance propionate production while simultaneously reducing CH_4 emissions, indicating potential compensation mechanisms.

Formate is used by many hydrogenotrophic rumen methanogens as an alternative substrate for methanogenesis [28] (Fig. 5). Our results revealed that the high formate concentration observed in the PEO group suggests that it could potentially be a less favorable alternative substrate for methanogens. It is likely that the electrons in formate can also be utilized in propionate synthesis rather than in methanogenesis, as suggested by Ungerfeld [29]. Additionally, we found a positive correlation between formate concentration and the Christensenellaceae R-7 group, which was enriched in the PEO group (Fig. 7). A recent study identified the Christensenellaceae R-7 group as a keystone species in the network, suggesting its importance in the rumen of yaks at various growth phases [30]. The identification of the Christensenellaceae R-7 group as a keystone taxon implies that it likely plays a pivotal role in shaping the overall structure and stability of the rumen microbial community. Therefore, understanding the specific roles and interactions of the Christensenellaceae R-7 group in the context of formate utilization and rumen fermentation could provide valuable insights into optimizing and mitigating CH_4 emissions in goats.

Methanomethylophilaceae is a newly proposed family within the order Methanomassiliicoccales [31]. It is capable of using trimethylamine to produce CH_4 [32]. Most trimethylamines are derived from choline in the rumen, which strongly inhibits CH_4 emission [33]. In the present study, the higher abundance of UG Methanomethylophilaceae in the CON group may be attributed to the lower concentration of trimethylamine, which could potentially be utilized as a source of CH_4. Ruminal fungi play a significant role in host energy metabolism by secreting large amounts of cellulolytic enzymes that are essential for

the degradation of structural polysaccharides [1]. In the present study, the fungal genera (e.g., *Piromyces* and *Neocallimastix*) were enriched in the CON group. Both *Piromyces* and *Neocallimastix* are major rumen fungal genera that possess a relatively great ability to utilize recalcitrant materials, such as cellulose and hemicellulose, owing to their production of cellulolytic and hemi-cellulolytic enzymes [34, 35]. The major fermentation end products are acetate, ethanol, formate, lactate, and succinate [36]. In contrast, *Methanobrevibacter* and *Caecomyces* (fungal genus) were positively correlated with CH_4 production (Fig. 7). These findings align with the predicted functional features, as the CON group displayed enriched CH_4 metabolism, and these two genera (*Methanobrevibacter* and *Caecomyces*) are known for their high CH_4 production capacity [37].

In the present study, PEO did alter microbial interaction and predicted functions. Starch and sucrose metabolism were enriched in the PEO group, playing a primary role for *Selenomonas*, which also exhibited a positive correlation co-occurrence with propionate (Fig. 8). Furthermore, succinate- and propionate-producing bacteria exhibited strong co-occurrence. This is consistent with previous studies on dairy cattle, where *Selenomonas* co-occurred with the family Succinivibrionaceae [38]. Given their ecological functions, it is plausible that they exhibit positive interactions and serve as pivotal bacteria in the rumen of highly efficient hosts [38]. Interestingly, the PEO group exhibits a lower concentration of propionate precursors. This can be attributed to the faster conversion of these precursors into propionate owing to the enrichment of propionate producers facilitated by PEO administration. This inference is supported by the enrichment of propionate metabolism in both the rumen microbiota and metabolites in the PEO group. The only positive correlation observed was between *Neocallimastix* and succinate, whereas *Anaerovibrio* showed a mutually exclusive relationship. *Anaerovibrio* is a typical lipid decomposer that produces both succinate and propionate, and serves as the keystone genus in the PEO group. Our results suggest that *Neocallimastix* in the PEO group does not play a crucial role in succinate production, which is a representative precursor of propionate. Ruminal fungi engage in synergistic interactions with bacteria and generate significant amounts of H_2, which serves as a primary substrate for methanogens and ultimately leads to the production of CH_4. Therefore, we speculate that *Piromyces* and *Neocallimastix*, considering their functional characteristics, may lead to enhanced CH_4 production owing to the improved cell wall degradation of fibrous ingredients [39]. Interestingly, most predicted functional enzymes were enriched in the PEO group. This may be attributed to the greater abundance of minor fungal genera in the PEO group that contribute to the secretion of a large variety of enzymes. Diverse rumen microbiota, including prokaryotic, protozoal, and fungal genera, co-occur with trimethylamines. The co-occurrence of trimethylamine with these microbiota suggests a potential synergistic effect. For instance, while some of these microbiota might produce trimethylamine, others might utilize trimethylamine as a substrate for their metabolism, providing UG Methanomethylophilaceae with more opportunities for utilization, resulting in increased CH_4 production. Furthermore, a positive correlation was observed between UG Methanomethylophilaceae and CH_4 metabolism in this study.

This study also identified the altered rumen taxa and interactions that can contribute to rumen metabolites and serum metabolites, suggesting although no phenotypes were different, the observed

metabolites may have positive and/or negative effect on goats' metabolism. For example, Zhao et al. [40] reported that administering sulfate or elemental sulfur (S) is an effective option for reducing ruminal CH_4 emissions by diverting ruminal H_2 away from CH_4 production. These sources are metabolized in the rumen by sulfur-reducing bacteria, such as *Desulfovibrio*, *Desulfohalobium*, and *Sulfolobus*, which can potentially accelerate sulfur metabolism and compete with methanogens [41, 42]. Interestingly, our results showed a higher enrichment of sulfur metabolism in the PEO group than in the CON group, whereas CH_4 metabolism was more enhanced in the CON group than in the PEO group. However, the abundance of *Desulfovibrio* did not differ significantly between the CON and PEO groups (CON, 0.45%; PEO, 0.37%; FDR, 0.675). Moreover, the only S-containing amino acid found in the rumen was methionine, and its concentration was similar in both the CON and PEO groups (CON, 5.84; PEO, 5.08; FDR, 0.845). Nevertheless, ruminal sulfur metabolism was highly enriched in the PEO group. Because sulfate or elemental S was not administered to either the CON or PEO groups, it is difficult to conclude that the specific enrichment of sulfur metabolism was indeed higher in the PEO group than in the current data. However, additional studies should be conducted to further investigate this phenomenon.

Additionally, many studies have shown that essential oil administration affects ruminal protein metabolism. In a previous study, the NH_3-N concentration was higher in the CON group in the growing phases of goats. This was due to the enrichment of NH_3-producing bacteria (such as Butyricicoccaceae, Clostridia UCG-014, and Prevotellaceae UCG-004) and N metabolism [16]. However, in the present study, we found no significant differences in the NH_3-N concentrations between the CON and PEO groups, indicating that PEO administration did not affect the abundance of NH_3-producing bacteria. We found that the composition of protozoal genera, which are crucial for rumen protein utilization, was not significantly different between the two groups. NH_3-N concentration showed a positive correlation with *Dasytricha* (protozoal genus). Moreover, blood metabolites (BUN and total protein) remained unaffected after PEO administration. These results provide strong evidence of their limited impact. Furthermore, we observed enriched amino acid metabolism (valine, leucine, and isoleucine degradation) in the CON group. Xue et al. [43] reported similar enrichment of this metabolism in cows with a microbiome characterized by low milk yield and protein content. In contrast, a high abundance of *Prevotella* could be helpful for the biosynthesis of valine, leucine, and isoleucine pathways. In line with this, our results indicated that the abundance of *Prevotella* was higher in the PEO group than in the CON group. Taken together, our results indicate that PEO administration does not significantly affect N metabolism during the fattening phase in goats.

While our study provides valuable insights into the effects of oral administration of PEO on rumen fermentation and microbial dynamics in goats, it is essential to acknowledge certain limitations that may have influenced our results. One notable limitation is the relatively small sample size used in our study, which might potentially affect the generalizability of our findings. Nonetheless, we employed a 2 × 2 crossover design to mitigate the impact of individual animal variations. For future multi-omics studies focused on investigating the microbial mechanisms contributing to host performance, it is advisable to contemplate a larger sample size.

Conclusions

This study examined the effect of PEO administration as a potential intervention to reduce enteric CH_4 emissions in goats. Our study found that PEO reduced CH_4 emissions by 12.0–13.6% ($P < 0.05$) during erucation. However, PEO did not affect animal performance or ruminal alpha and beta diversity of bacteria, archaea, protozoa, or fungi. The co-occurrence and mutual exclusion patterns of some genera were altered following PEO administration. Notably, the enrichment of *Selenomonas* in the PEO group likely contributed to enhanced propionate metabolism in the rumen. The predicted functional features revealed that starch, sucrose, sulfur, and propionate metabolism was enriched in the PEO group, whereas CH_4 metabolism was enriched in the CON group. Random forest analysis identified eight rumen metabolites that could potentially serve as metabolic markers following PEO administration. Among these, the propionate concentration exhibited a positive correlation and co-occurrence relationship with *Selenomonas*. Importantly, our results suggest that PEO administration employs diverse mechanisms of action, such as sulfur, starch, sucrose, trimethylamine, and propionate, collectively working to enhance CH_4 inhibition, while also providing alternative H_2 sinks. Further research is warranted to specifically examine how the reduction in CH_4 emissions through PEO administration correlates with improved milk and meat production efficiency in goats, offering valuable insights into optimizing their overall productivity while minimizing environmental impact.

Methods

Animal ethics statement

The experimental procedures were reviewed and approved by the Gyeongsang National University Institutional Animal Care and Use Committee (protocol number: GNU-210705-E0063). The experiment took place at the Gyeongsang National University Animal Breeding Farm from November 25, 2022, to February 9, 2023.

Experimental design, animals and diet

A total of 10 fattening Korean native goats (*Capra hircus coreanae*, 42.3 ± 1.68 kg, male) were kept in individual pens (170× 120 cm) and were randomly divided into two dietary groups: (1) control (CON; basal diet without additive) and (2) PEO, basal diet + 1.5 g/d of PEO) using a 2 × 2 crossover design. The PEO added in this study was in liquid form and extracted from *Pinus koraiensis* pinecones, which were provided by PHYLUS (PHYLUS Co., Ltd. Seoul, Korea). The detailed information regarding the PEO extraction process and its constituents is fully described in our previous paper [15].

The orally administration dosages of PEO were determined based on our previous study [16]. To ensure the goats received the full PEO dose, we orally administered PEO aliquots in 5 mL of water using a 10 mL syringe. The CON group was given 5 mL of water. All animals were fed the same diet, consisting of tall fescue and a commercial concentrate. The chemical composition of the tall fescue and commercial

concentrate are presented in Table 1. The animals were given their diet and PEO additives in two equal meals at 0800 h and 1600 h. The experimental diet included a mixture of tall fescue hay and concentrate in a 50:50 ratio, meeting the nutrient requirements based on NRC (2007) recommendations. Before providing the concentrate mix, tall fescue hay was given to encourage the goats to consume as much forage as possible. Drinking water was available at all times. The individual daily feed intake was recorded by measuring both the feed offered and any refusals. Each experimental period lasted for 28 days, with 23 days of adaptation followed by 5 days of data and sample collection. Additionally, there was a 21 days wash-out period between the two experimental periods.

Table 1
Mineral and chemical composition of experimental diets fed to goats.

Item[1]	Tall fescue	Concentrate[2]
Mineral composition (% of DM)		
Ash	8.21	9.46
Calcium	0.25	1.25
Phosphorus	0.19	0.66
Magnesium	0.18	0.32
Potassium	2.36	1.24
Sodium	0.04	0.36
Iron, mg/kg	159	366
Manganese, mg/kg	40.0	128
Zinc, mg/kg	20.0	174
Copper, mg/kg	6.00	34.0
Chemical composition (% of DM)		
Dry matter	92.9	89.5
Moisture	7.10	10.5
Crude protein	11.6	16.4
Soluble protein	4.50	6.22
Ether extract	1.52	4.14
Starch	1.23	-

SEM: standard error of the mean; DM: dry matter. NDF: neutral detergent fiber; NDICP: neutral detergent insoluble crude protein; ADF: Acid detergent fiber; ADICP: acid detergent insoluble crude protein.

[1]Chemical analysis was performed by Cumberland Valley Analytical Services (Waynesboro, PA, USA).

[2]The formulation of the concentrate used in this study contained crude protein (13.5% or more), ether extract (2.5% or more), crude fiber (20.0% or less), ash (10.0% or less), calcium (0.80% or more), phosphate (0.80% or less), and TDN 67% (Values are concentrations declared by the manufacturer).

[3]According to Hall (2000) equation.

[4]Net energy for maintenance was calculated using the OARDC Summative Energy Equation of Weiss (1998).

Item[1]	Tall fescue	Concentrate[2]
NDF	48.6	33.7
NDICP	1.20	4.37
ADF	26.8	16.6
ADICP	0.66	1.25
Lignin	2.18	4.50
Non-fiber carbohydrates[2]	31.3	36.3
Energy, Mcal/lb[3]		
Metabolizable energy	0.67	1.17
Net energy maintenance	0.71	0.76
Net energy gain	0.44	0.49
Total digestible nutrients (% of DM)	65.2	68.2

SEM: standard error of the mean; DM: dry matter. NDF: neutral detergent fiber; NDICP: neutral detergent insoluble crude protein; ADF: Acid detergent fiber; ADICP: acid detergent insoluble crude protein.

[1]Chemical analysis was performed by Cumberland Valley Analytical Services (Waynesboro, PA, USA).

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[3]According to Hall (2000) equation.

[4]Net energy for maintenance was calculated using the OARDC Summative Energy Equation of Weiss (1998).

Nutritional analysis of feed

Dried feed samples (tall fescue and concentrate) were ground through a 1 mm sieve using a Wiley Mill (Arthur Thomas CO., Philadelphia, PA). The ground samples were sent to Cumberland Valley Analytical Services Inc. (Waynesboro, PA) for wet chemistry analysis. The analysis included measurements of dry matter (DM), crude protein (CP), ether extract (EE), ash, minerals, amylase-treated neutral detergent fiber (aNDF), acid detergent fiber (ADF), neutral detergent insoluble crude protein (NDICP), acid detergent insoluble crude protein (ADICP), lignin, and starch. The respective analysis methods used were referenced as follows: DM (AOAC International, 2000 [44]; method 930.15), CP (AOAC International, 2000; method 990.03), EE (AOAC International, 2006 [45]; method 2003.05), ash (AOAC International, 2000; method 942.05), minerals (AOAC International, 2000; method 985.01), aNDF (Van Soest et al., 1991), ADF (AOAC

International, 2000; method 973.18), NDICP and ADICP (analyzed using Leco FP-528 N Combustion Analyzer lignin [46], and starch [47]. Non-fiber carbohydrate (NFC) were calculated using Hall's equation [48]; NFC = 100 – [(CP – NDICP) + EE + ash + NDF]. The OARDC Summative Energy Equation, as described by Weiss [49], was utilized for calculating the net energy for maintenance.

Methane measurements and data processing

Enteric CH_4 emissions were quantified using a laser methane detector (LMD) (LMm-G; Tokyo Gas Engineering Co. Ltd, Tokyo, Japan) following the procedure described by Roessler et al. [50] and Kang et al. [51] with minor modifications. In brief, CH_4 emissions were measured twice a day during four consecutive days (24–27 days), specifically before feed intake (0600–0800 h) and after feed intake (0900–1100 h). The CH_4 concentration in the breathing air was continuously monitored at an interval of 0.5 s for a duration of 8 min, recorded in ppm-m. Eructation, which is the main source of CH_4 emissions, typically occurs during the B-sequence of rumen contractions. These contractions happen irregularly, with a frequency of approximately once every 1–3 min [52]. To adequately capture CH_4 emissions, the measurement duration was set to 8 min, as recommended by Kang et al. [51], which allowed for the detection of 3–4 eructation events. The detailed measurement method is fully described in our previous paper [16].

The measured CH_4 concentrations were determined using method described by Kang et al. [51], where the *AMPD* package in R was used to identify the peaks in the measured CH_4 concentration data obtained from LMD. The data peaks were separated into two categories (respiration and eructation) using the *mixdist* R package, and each category was analyzed separately. The mean of the normal distribution was assumed to be the representative CH_4 concentration of the gas exhaled from the track for the hour. The values of CH_4 concentration measured four times a day were averaged to represent the mean daily CH_4 concentration. Daily CH_4 emission data were averaged to report one value per goat per period, and data from goats with less than 2 d of successful measurements were excluded from statistical analysis.

Sample collection and analyses

1. Rumen fluid sampling

Before morning feeding, rumen contents were collected from each animal using oral stomach tubing (length of 150 cm and a diameter of 0.8 cm). To reduce saliva contamination, the first 20 mL of each rumen fluid sample was discarded. Subsequently, collected rumen fluid from each goat was filtered through 4 layers of cheesecloth and measured their pH with a pH meter (S220, Mettler-Toledo, Greifensee, Switzerland). After filtering the rumen fluid (10 mL), it was divided into two separate aliquots for volatile fatty acid (VFA) and ammonia nitrogen (NH_3-N) analysis. Another 5 mL of filtered rumen fluid was centrifuged at 20,000×*g* for 10 min at 4 ℃. The supernatant was discarded, and the pellet was stored at -80 ℃ for microbial analysis. All aliquots were transported to the laboratory (with dry ice) and stored at -80 ℃ until further analysis.

2. Rumen fermentation parameters analyses

For VFA processing, rumen fluid samples of 1 mL were subjected to centrifugation at 20,000×*g* for 10 min at 4 ℃. The resulting supernatant was utilized for analysis using a high-performance liquid chromatography system (L-2200, Hitachi, Tokyo, Japan) equipped with a UV detector (L-2400; Hitachi) and a column (MetaCarb 87H; Varian, Palo Alto, CA, USA). Concentration of the NH_3-N was determined using a spectrophotometer (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) by measuring the optical density at 630 nm. The measurement was conducted following the protocol described by Chaney and Marbach [53].

3. Blood sampling and analyses

On d 28 of each sampling period before the morning feeding, blood from the jugular neck vein was collected in a serum-separating tube (BD Vacutainer, SSTTM advance, Becton Dickinson Co., Franklin Lakes, NJ, USA) from goats. The blood samples were centrifuged for 15 min at 1,006×*g* at 4 ℃, and the serum was stored at -80 ℃ until analysis. The content of serum alanine transaminase/serum glutamic pyruvate transaminase (ALT/SGPT), aspartate aminotransferase/serum glutamic oxaloacetic transaminase (AST/SGOT), blood urea nitrogen (BUN), inorganic phosphate, and glucose were measured using the UV spectrophotometry method by a Cobas 8000 c702 analyzer (Roche Diagnostics, Mannheim, Germany). The contents of albumin, total cholesterol, creatinine, total protein, triglyceride, and calcium were measured using the colorimetry method by a Cobas 8000 c702 analyzer (Roche Diagnostics, Mannheim, Germany).

4. Fecal sampling and analysis

To estimate DM digestibility, fecal samples were collected thrice on a 12-hour basis during each experimental period. Within 6 h of excretion, each fecal sample was weighed and placed in individual bag. The fecal samples were dried in a forced-air oven at 55 ℃ for 96 hours until their weight stabilized. The detailed information regarding the fecal sampling process is fully described in our previous paper [16].

5. Multi-kingdom amplicon library preparation and sequencing analyses

Total DNA of rumen fluid (1.8 mL) was extracted using the repeat bead-beating plus column method [54]. The quality and quantity of extracted DNA were evaluated using a NanoDrop spectrophotometer (ND-2000, Thermo Fisher Scientific Inc., Waltham, MA, USA). Rumen microbiota was analyzed using metataxonomic based on kingdom-specific phylogenetic markers (16S rRNA gene for bacteria and archaea [55], 18S rRNA gene for protozoa [56], and internally transcribed spacer 1 (ITS1) region for fungi [57]). Extracted gDNA was submitted to Macrogen (Seoul, Korea) for library preparation each primer regions (**Table S1**). Preparation of the amplicons barcoded library was based on the Illumina 16S, 18S, and ITS1 amplicon sequencing library preparation protocol and the sequencing was performed using

Illumina MiSeq platSform (San Diego, CA, USA). The obtained amplicon sequencing data were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2, v. 2021.11; [58]).

Briefly, adapter and primer sequences of bacteria and protozoa were removed using Cutadapt [59] followed by quality filtering (Q-score > 20), denoising, merging, and chimeric sequence removal as done previously using q2-dada2's denoise-pair method [60]. For the fungal ITS1 sequences, the reads were quality-filtered using the q2-dada2's denoise-single method [60]. Amplicon sequence variants (ASVs) were clustered at 99% similarity. Taxonomic classifiers for each kingdom were manually trained using the Naïve Bayes classifier [61] with the Silva (SSU138) gene database for bacteria and archaea and the UNITE ITS reference database (v.8.3. 05.10.2021 database) for fungi [62]. To classify protozoal ASVs, a BLASTn search was performed against the NCBI nucleotide collections (excluding uncultured/environmental samples accessed on May 3, 2023). The taxonomy of each ASV was then determined by selecting the best BLASTn hit. Major phyla and genera each representing ≥ 0.1% of total sequences in at least one of the dietary treatments were discussed in this report.

6. Analysis of sequencing data

Analysis of the differential relative abundances of rumen prokaryotes, protozoa, and fungi was evaluated using Microbiome Multivariable Associations with Linear Models (MaAsLin2) package in R [63]. Differential abundance was calculated using Centered Log-Ratio normalization and LM method, with experimental period and treatment as fixed effects and individual animal as random effects.

We excluded ASVs identified as unassigned, mitochondria, and chloroplast before downstream analysis. To reduce the sampling heterogeneity, the ASV table was rarefied to the same reads per sample (ASVs) with 1,000 times using the 'q2-repeat-rarefy' plugin from QIIME2 [64]. Microbial diversity was evaluated within samples (alpha diversity) or between samples (beta diversity) on rarefied ASVs table. Alpha diversity was evaluated using richness (Chao1 estimates), Evenness, Simpson's index, and Shannon's index. Beta diversity was evaluated using phylogenetic distance of Bray Curtis and Weighted UniFrac. Prediction of the metabolic functions of the rumen microbial communities was performed using PICRUSt2 (v.2.4.1) [65] and CowPI [66] to predict the functional profile of the microbial communities based on the 16S rRNA gene sequences obtained. Since the web server for CowPI is unavailable, the tool was reconstructed using precalculated files deposited on Zenodo (https://zenodo.org/record/1252858). Since MaAsLin2 did not detect predicted functional feature differences in the CowPI and PICRUSt2, linear discriminant analysis effect size (LEfSe) analysis was employed to find differences [67].

To understand relationship among the bacteria and archaea, protozoa, and fungi (relative abundance ≥ 0.1%) in the CON and PEO groups, the co-occurrence network analysis was generated using the 'FastSpar' [68] which use SparCC algorithm [69]. The raw amplicon sequences from this study were deposited in the NCBI Sequence Read Archive (accession numbers: PRJNA975721).

7. Metabolomic analysis using proton nuclear magnetic resonance (^{1}H-NMR)

Rumen fluid samples for the metabolite analysis were centrifuged it at 12,902×*g* for 10 min at 4 ℃, resulting in the collection of 300 µL of the supernatant. Subsequently, 300 µL of a standard buffer solution containing (2,2,3,3-d(4)-3-(trimethylsilyl) propionic acid [TSP] sodium salt) in deuterium oxide (D_2O) solvent/standard buffer solution (300 µL) was added to the supernatant. The combined supernatants (600 µL) were transferred to 5 mm NMR tubes for ^{1}H-NMR spectroscopy analysis. This sample pre-treatment was following the procedures described by Saleem et al. [70]. Similarly, serum samples for the metabolite analysis were centrifuged at 14,000×*g* for 10 min at 4 ℃. The supernatant (200 µL) was added to 400 µL of saline buffer (NaCl 0.9% w/v in 100% D_2O) in 5 mm NMR tube for ^{1}H-NMR spectroscopy analysis [71]. The spectra of rumen fluid and serum were acquired using SPE-800 MHz NMR-MS spectrometer (Bruker BioSpin AG, Fällanden, Switzerland) equipped with a 5 mm triple-resonance inverse cryoprobe featuring Z-gradients (Bruker BioSpin Co., Billerica, Massachusetts, USA). The Carr-Purcell-Meiboom-Gill pulse sequence was employed for acquiring the NMR spectra of rumen fluid and serum. Data acquisition involved collecting 64,000 data points with 128 transients, using a spectral width of 16,025.641 Hz, a relaxation delay of 4.0 s, and an acquisition time of 2.0 s [71].

8. Metabolomics data processing and analyses

The analyzed spectral data was utilized for metabolites identification and quantification using the Chenomx NMR suite 8.4 software (ChenomxInc, Edmonton, Alberta, Canada). The classification process involved the utilization of three metabolite databases: Bovine Metabolome Database (www.bovinedb.ca), the Livestock Metabolome Database (www.lmdb.ca), and the Human Metabolome Database (www.hmdb.ca). Statistical analyses of the metabolite data were performed using MetaboAnalyst 5.0 (http://www.metaboanalyst.ca). The obtained data were processed using normalization-selected methods, involving sample normalization with constant sum, data transformation through log normalization, and data scaling through auto scaling. The rumen fluid and serum metabolite data with 50% of samples under the identification limit or with at least 50% of values missing were eliminated from the analysis. Metabolic pathway analysis utilized the Bos taurus pathway library from the Kyoto Encyclopedia of Genes and Genomes (KEGG) website (http://www.kegg.com).

The “randomForest (RF)” package in R was used for the RF analysis [72]. The rumen metabolites were used as inputs in the RF model. For each metabolite, a mean decrease accuracy score was calculated based on the increase in error caused by removing that metabolite from the predictors. This score reflects the importance of metabolites in the model. The best predictive model was identified based on the maximum area under the curve (AUC), using the “pROC package” in R [73]. To minimize potential overfitting, we applied a 10-fold cross-validation approach using the "trainControl" package in R [74].

To predict the probabilities of co-occurrence between microbial genera and metabolites in host rumen fluid, we employed microbe-metabolite vectors (mmvec) neural network-based approach, which infers the

nature of interactions across omics datasets [75]. The interactions between microbes and metabolites were ranked and visualized through the standard dimensionality reduction interface that is implemented as a plugin in QIIME2 (Version 2021.2).

Statistical analysis

Statistical analyses were performed using SAS (v. 9.4, SAS Institute Inc., Cary, NC) and R software (v. 4.0.2). The normality of data was investigated with a Shapiro–Wilk test prior to all statistical analyses. The data obtained from *in vivo* experiment was analyzed using PROC GLIMMIX procedure according to the following statistical model:

$$Y_{ijk} = \mu + A_i + P_{j+} + T_k + (PT)_{jk} + \varepsilon_{ijk}$$

where, Y_{ijk} = observed dependent variable, μ = overall mean, A_i = random effect of animal, P_j = fixed effect of period, T_k = fixed effect of treatment, $(PT)_{jk}$ = fixed effect of interaction between period and treatment, and ε_{ijk} = unexplained error. The Wilcoxon rank-sum test was utilized for data with an abnormal distribution, and the *P* values were subsequently adjusted using FDR correction. The resulting distance matrices served as inputs for principal coordinates analysis (PCoA) and significance of sample clustering was analyzed by permutational multivariate analysis of variance (PERMANOVA) with 9,999 permutations. Regarding MaAsLin2, Benjamini-Hochberg false discovery rate (FDR, [76]) adjusted *Q*-values < 0.1 were considered as significant. LEfSe uses a nonparametric factorial Kruskal Wallis and Wilcoxon rank sum test followed by a linear discriminate analysis to estimate the effect size of each taxon. A significance level of $P < 0.05$ and effect size threshold of 2 were applied in the trial to identify the biomarker functional features. Comparison of each exclusive networks were accomplished with Co-expression Differential Network Analysis (CoDiNA) [77]. To define network statistics, we used the built-in plugins in Gephi (v. 0.9.2) [78] to calculate measurements of centrality (i.e., eigenvector centrality and authority). The univariate Student's t-test was used to quantify differences between in the metabolite profiles of the rumen fluid and serum under the CON and PEO groups. Afterward, metabolite profiles resulting from the analysis of NMR data were used as explanatory variables in a partial least squares discriminant analysis (PLS-DA) to investigate how they contributed to difference after PEO administration. The variable importance in projection method (VIP) was applied to assess the relevance of each metabolite considering the ordinary VIP ≥ 1.5 threshold, in order to identify the most important features. *P* values were corrected for FDR [76] and $P < 0.05$ and $0.05 \leq `P < 0.01$ were considered as significant and tendency effects, respectively.

Abbreviations

ALT/SGPT: Alanine transaminase/serum glutamic pyruvate transaminase

AST/SGOT: Aspartate aminotransferase/serum glutamic oxaloacetic transaminase

BW: Body weight

CH_4: Methane

CON: Control

DDMI: Digestible dry matter intake

DM: Dry matter

DMD: Dry matter digestibility

DMI: Dry matter intake

FDR: False discovery rate

H_2: Hydrogen

NFC: Non-fiber carbohydrate

NH_3-N: Ammonia nitrogen

PCoA: Principal coordinates analysis

PERMANOVA: Permutational multivariate analysis of variance

RF: Random forest

VFA: Volatile fatty acid

VIP: Variable importance in projection

Declarations

Ethics approval and consent to participate

The experimental procedures were reviewed and approved by the Gyeongsang National University Institutional Animal Care and Use Committee (protocol number: GNU-210705-E0063). All authors have consented to participate.

Consent for publication

All authors have consented to publication.

Availability of data and materials

All data presented in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author's contributions

Y.C. and **S. J. L.** wrote the main manuscript text, prepared tables and figures, and performed amplicon sequencing data analysis. **S. J. L.** provided funding. **H. S. K.** and **J. S. E.** assisted metabolomic analysis and assisted in drafting of the manuscript. **S.U.J.** conducted a thorough statistical analysis of the measured methane data. **L.L.G.** reviewed and edited the manuscript. **S.S.L.** was responsible for experimental concept and design, supervised all work, and critically revised the manuscript. All authors reviewed the manuscript.

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Figures

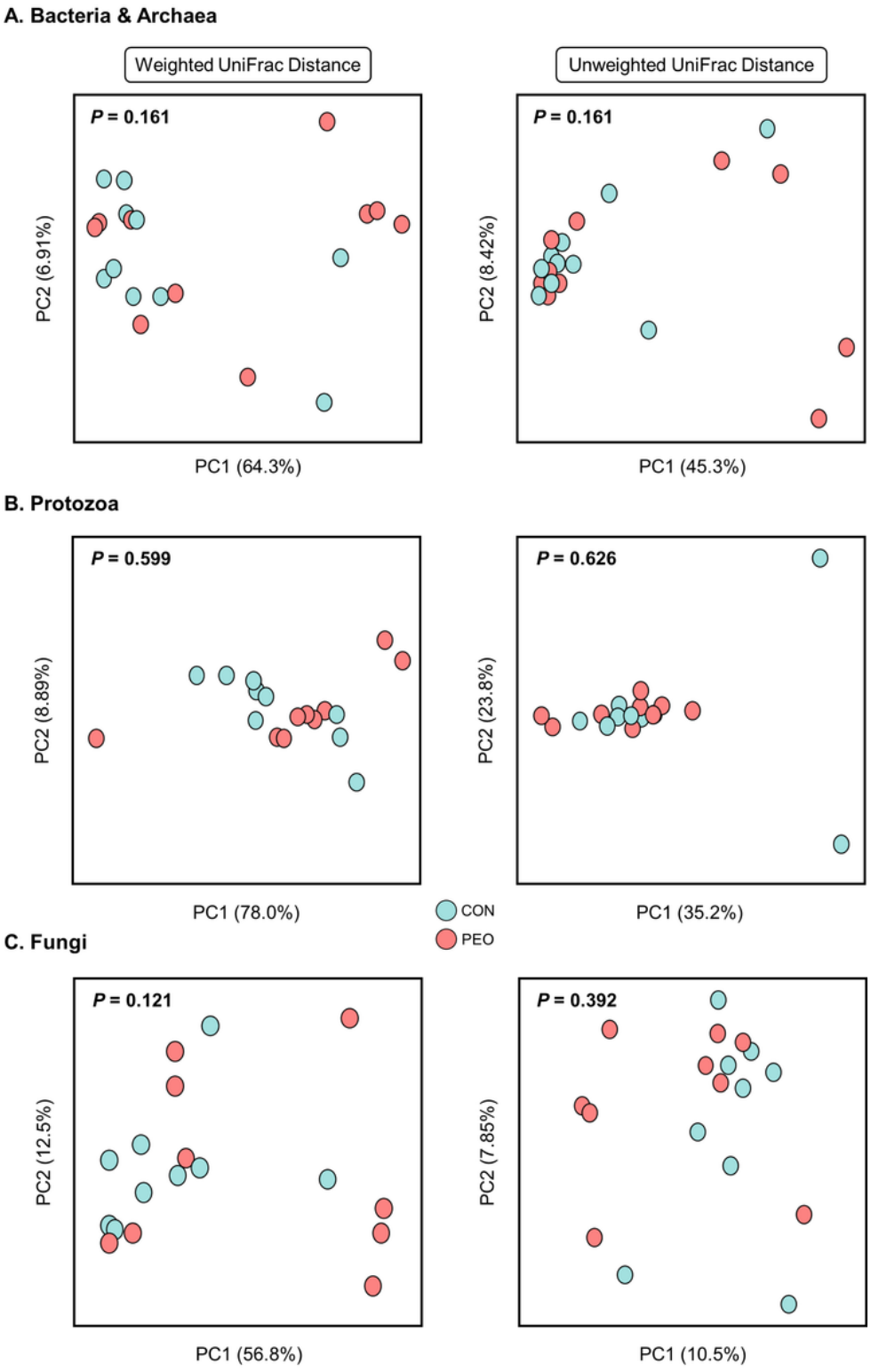

Figure 1

Principal coordinate analysis (PCoA) of the ruminal microbiota of **A** bacteria and archaea, **B**protozoa, and **C** fungi based on the matrices of Weighted UniFrac distance and Unweighted UniFrac distance. CON, without PEO; PEO, *Pinus koraiensis* cone essential oil.

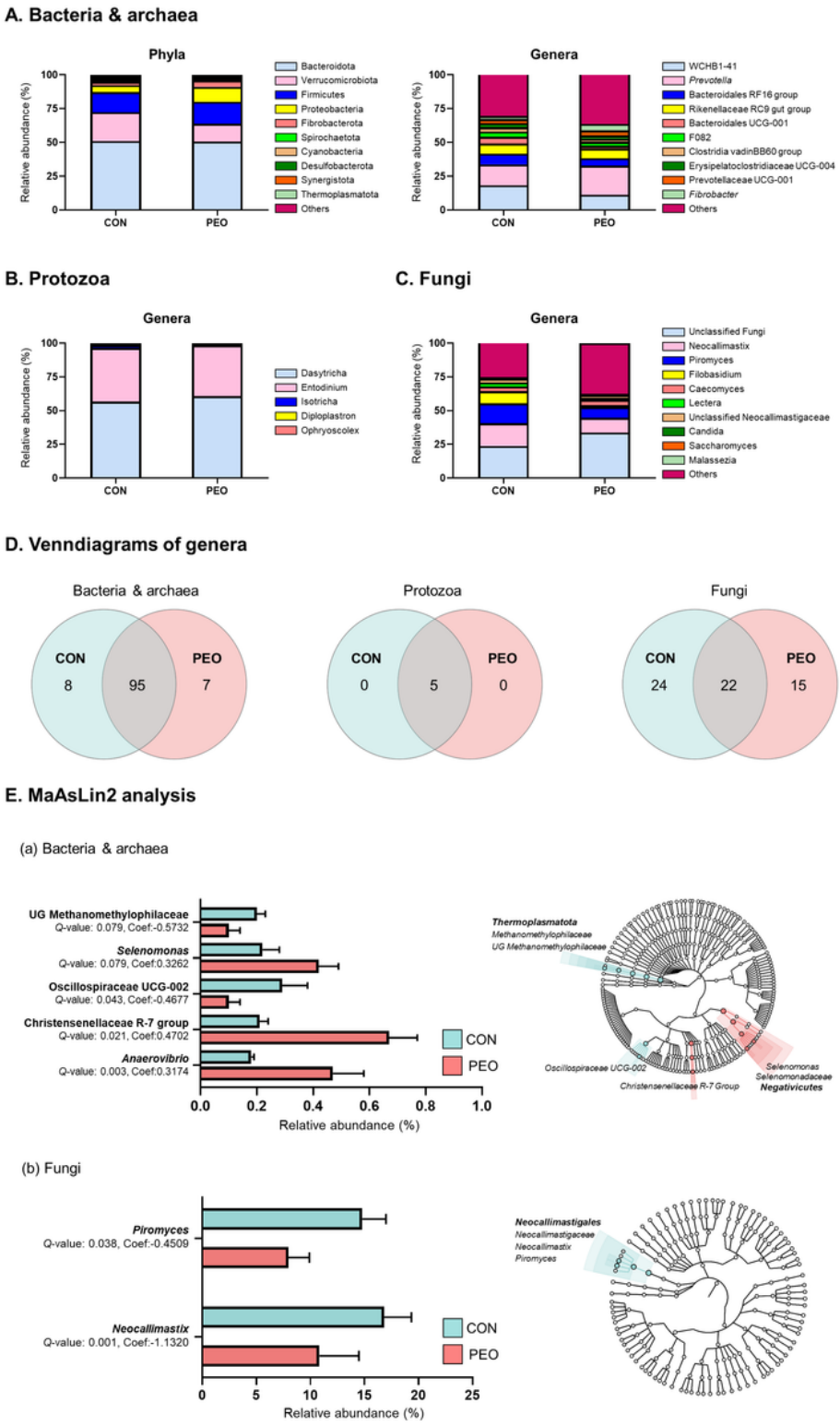

Figure 2

Compositional profiles of ruminal microbiota in goats, including **A**bacteria and archaea, **B** protozoa, and **C** fungi. **D** Venn diagrams showing the genera of rumen microbes shared between and unique to the CON and PEO group. **E**Horizontal barplots showing the genera associated with the PEO group, compared to the CON group, as detected by MaAsLin2. Genera with Benjamini-Hochberg false discovery rate-adjusted $Q <$ 0.1 were considered statistically significant for (a) bacteria and archaea, and (b) fungi. Relative

abundance of major phyla and genera (relative abundance ≥ 0.1% in more than 50% animals) for all individuals. CON, without PEO; PEO, *Pinus koraiensis* cone essential oil; Coef, coefficient; *Q*-value, *P* value corrected by the Benjamini-Hochberg method; MaAsLin2; microbiome multivariable association with linear models.

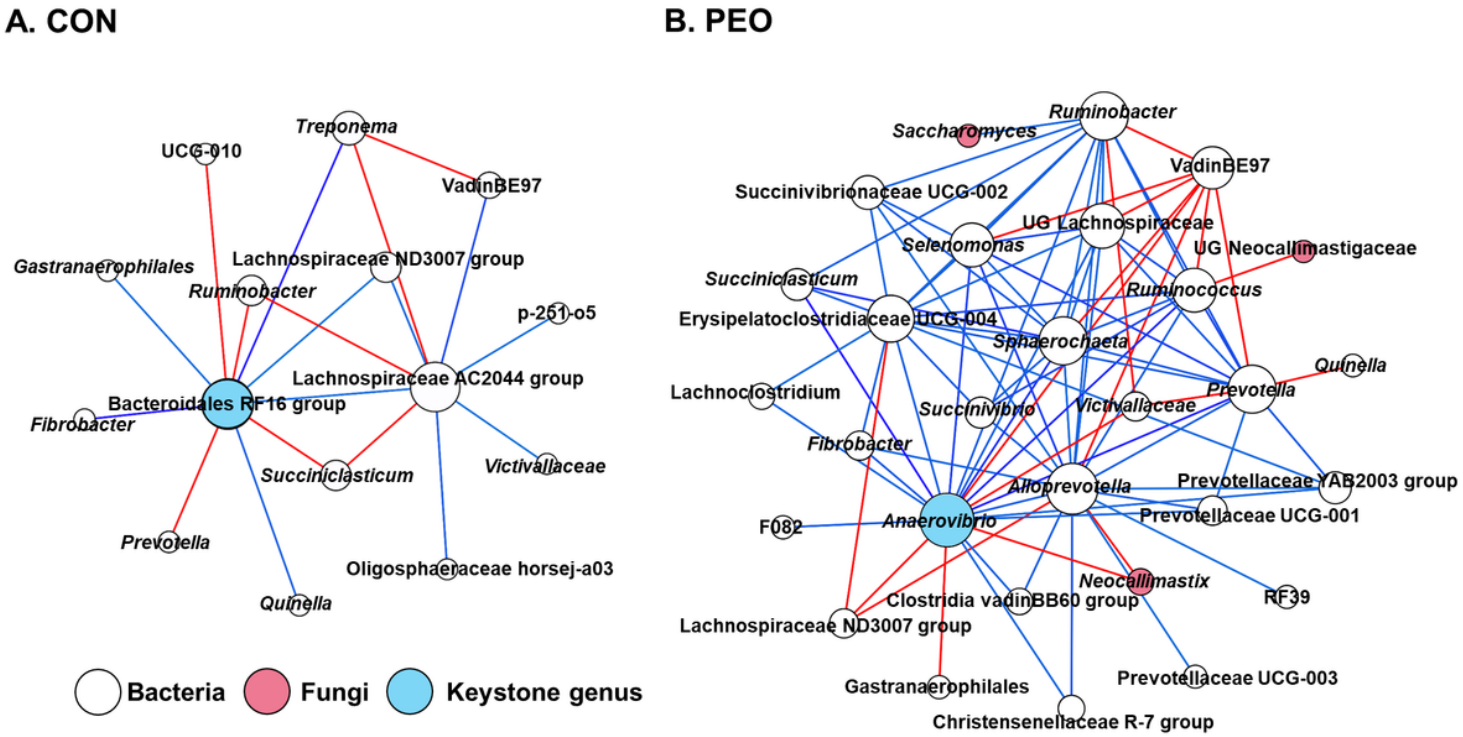

Figure 3

Exclusive co-occurrence and mutual exclusion microbial network in **A** CON and **B** PEO oral administration. The node color represents bacteria (white), fungi (pink), and keystone genus (skyblue). The keystone genus is selected based on authority and eigenvector centrality measurements within each exclusive network. The edge color represents co-occurrence (blue) or mutual exclusive (red) interactions. The thickness of the edges is adjusted based on the absolute value of the correlation coefficients of each interaction. Only genera accounting for ≥ 0.1% average relative abundance in at least one of the treatments were used. CON, without PEO; PEO, *Pinus koraiensis* cone essential oil.

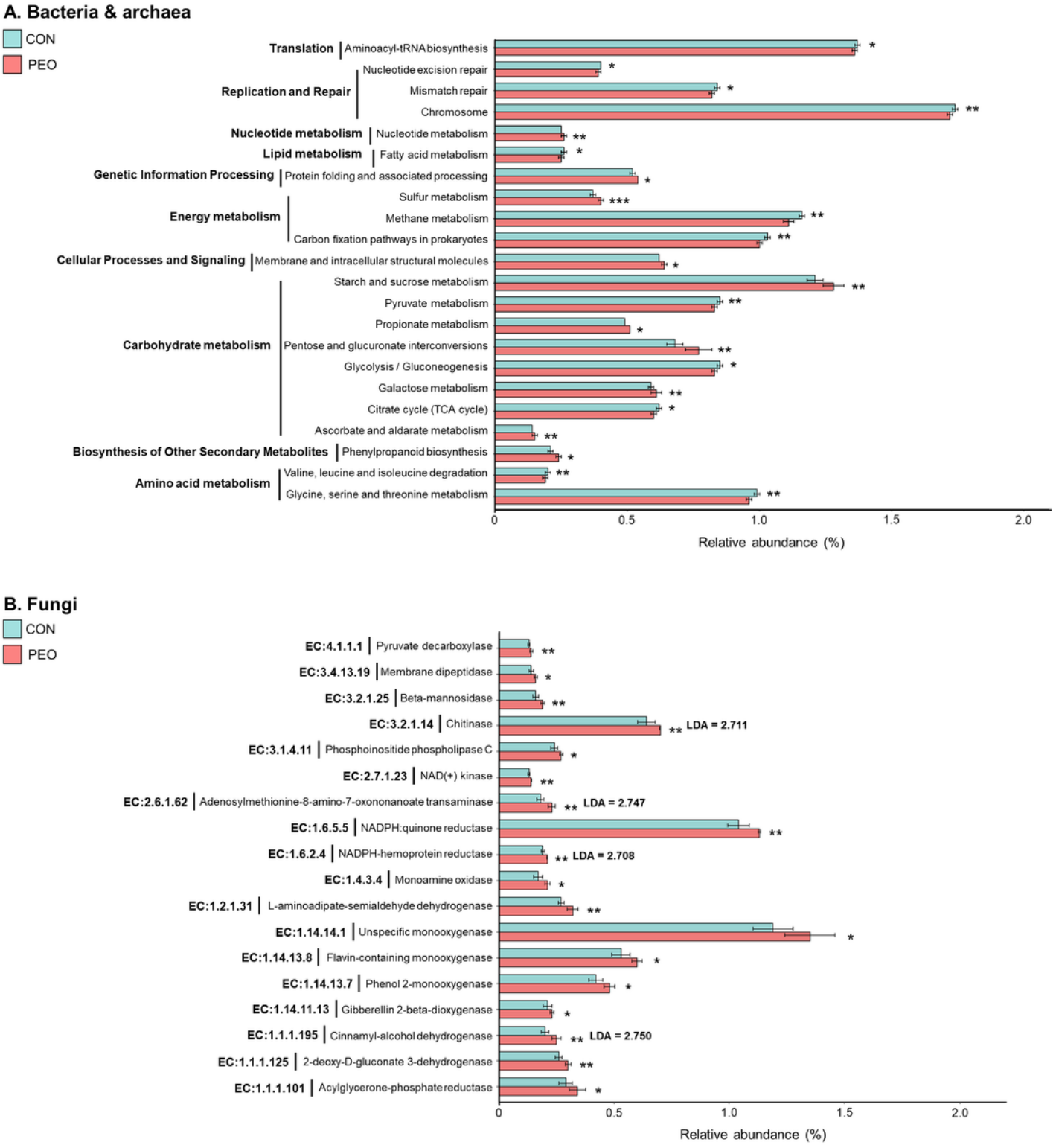

Figure 4

Predicted **A** prokaryotic functions (CowPI database) and **B** fungal enzymes (PICRUSt2 database) detected using LEfSe (LDA > 2.0, $P < 0.05$) in the ruminal microbiota of CON and PEO groups. Only the functional parameters accounting for ≥ 0.1% average relative abundance in at least one of the treatments were statistically analyzed by LEfSe. CON, without PEO; PEO, *Pinus koraiensis* cone essential

oil; LDA: linear discriminant analysis; LEfSe: linear discriminant analysis effect size. *P< 0.1, **P < 0.05, ***P < 0.001.

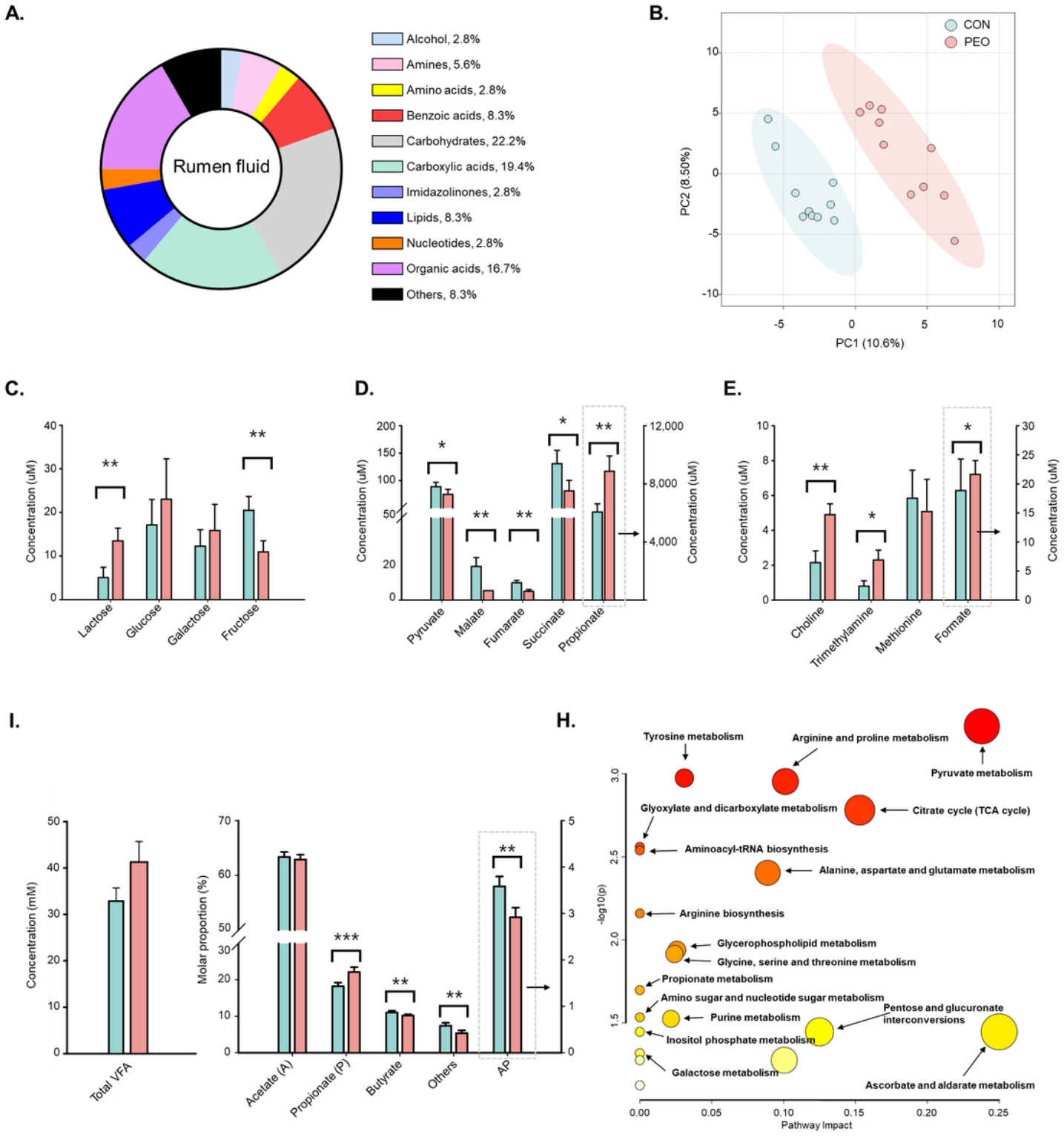

Figure 5

A Classification of measured metabolites according to chemical class in rumen fluid. **B** Partial least square discriminant analysis (PLS-DA) score plot of rumen fluid. **C** Concentration of carbohydrates, **D**

concentration of propionate precursors and propionate, **E**concentration of choline, trimethylamine, and formate, **I** concentration of total VFA, molar proportions of individual VFAs, and AP ratio. **H** Metabolic pathway mapping of common quantified metabolites in the rumen fluid. Selected metabolites met the criteria of $P < 0.1$ and VIP score ≥ 1.5. CON, without PEO; PEO, *Pinus koraiensis* cone essential oil; VFA, volatile fatty acid; AP, acetate to propionate; VIP, variable importance in projection. *$P < 0.1$, **$P < 0.05$.

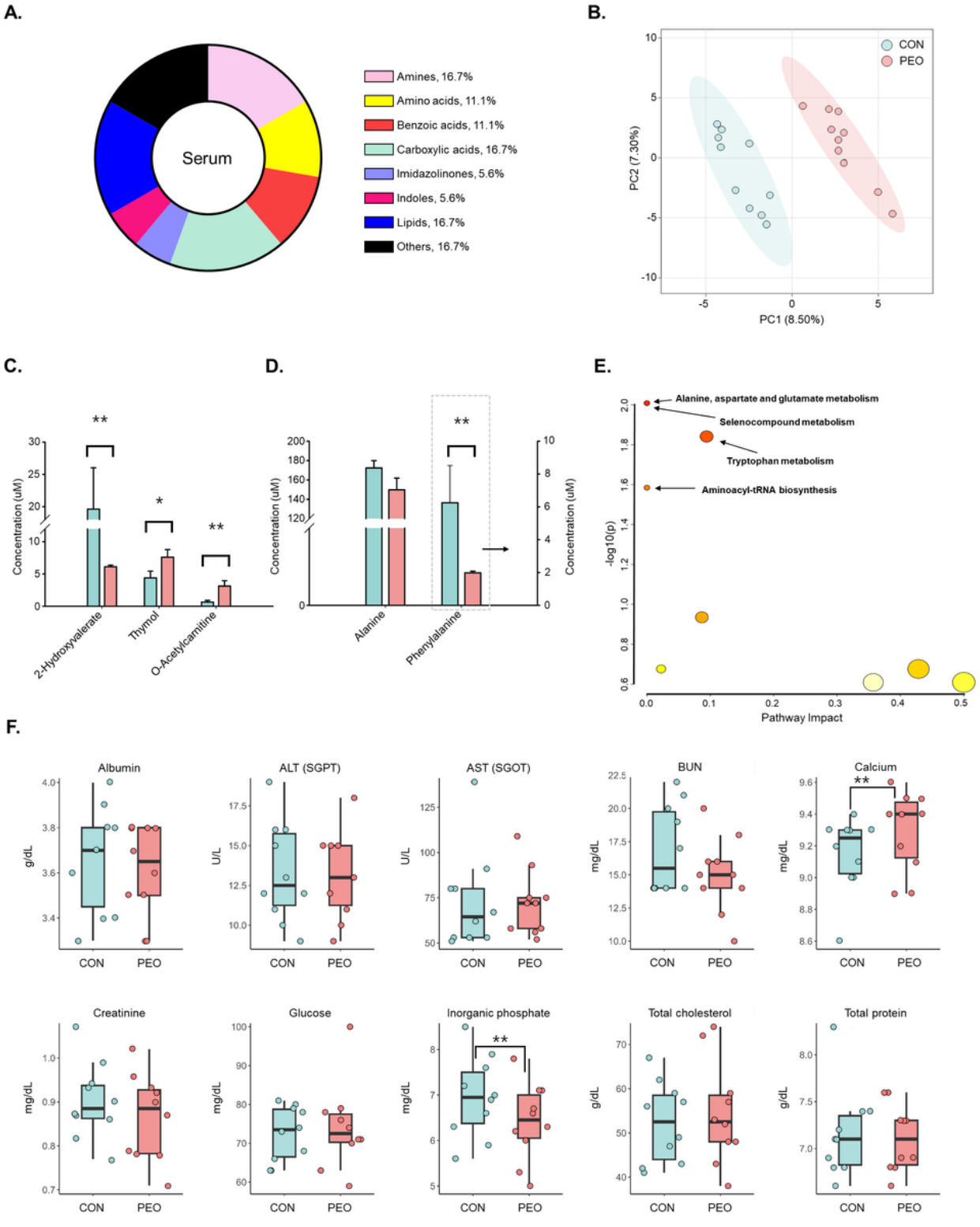

Figure 6

A Classification of measured metabolites according to chemical class in serum using ^{1}H-NMR. **B** Partial least square discriminant analysis (PLS-DA) score plot of serum. **C** Concentration of lipids and **D** amino acids. **E** Metabolic pathway mapping of common quantified metabolites in the serum. **F** Concentration of serum metabolites and liver enzymes using UV spectroscopy and colorimetry method. Selected metabolites obtained from ^{1}H-NMR met the criteria of $P < 0.1$ and VIP score ≥ 1.5. CON, without PEO; PEO, *Pinus koraiensis* cone essential oil; ALT/SGPT, alanine transaminase/serum glutamic pyruvate transaminase; AST/SGOT, aspartate aminotransferase/serum glutamic oxaloacetic transaminase; BUN: blood urea nitrogen. *$P < 0.1$, **$P < 0.05$.

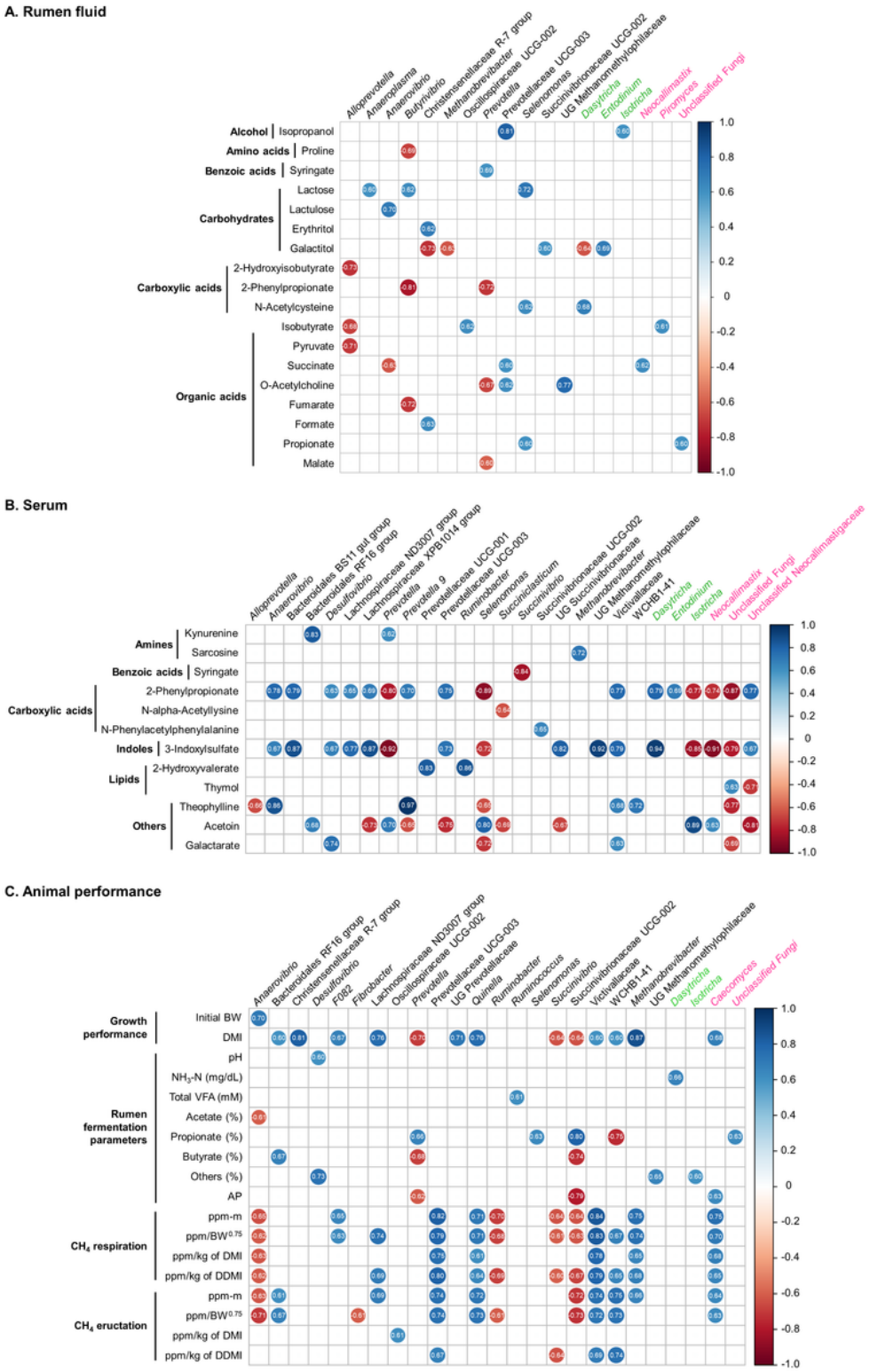

Figure 7

Correlation of **A** rumen and **B** serum metabolites, and **C** animal performance parameters with the relative abundance of major bacterial and archaeal, protozoal (green), and fungal (pink) genera (occupying over 0.1% average relative abundance in at least one of the treatments). Correlation analyses were conducted using Spearman's rank correlation. Only strong correlation coefficients ($|r| \geq 0.6$) and significant ($P < 0.05$) correlations were selected to be shown on the plot. BW, body weight; CH_4, methane; DMI, dry matter

intake; DDMI, digestible dry matter intake; NH_3-N, ammonia nitrogen; Others, valerate, isovalerate, and isobutyrate; AP, acetate to propionate.

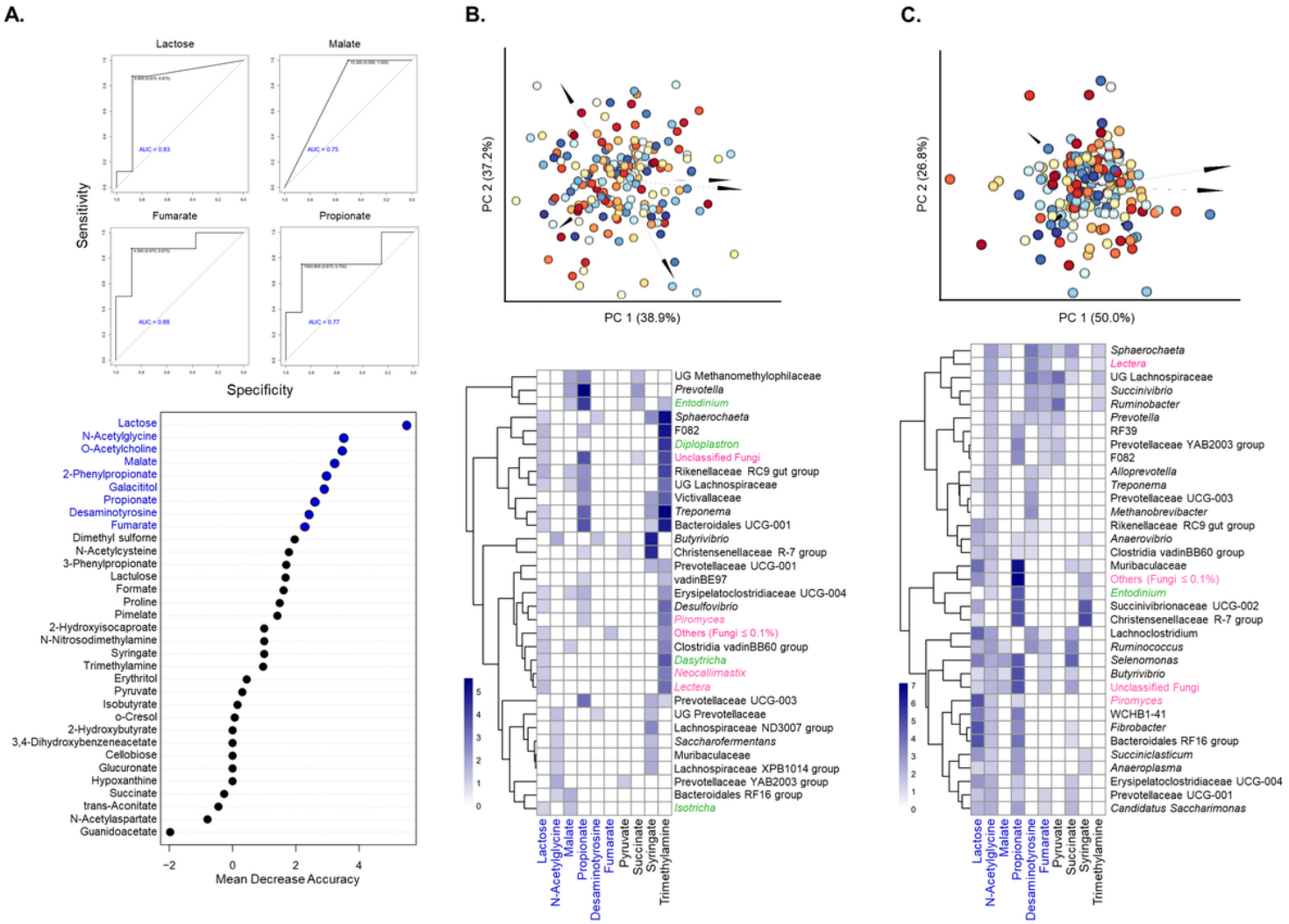

Figure 8

Prediction of microbe and metabolite co-occurrences in Korean native goats between CON and PEO group. **A** Receiver operating characteristic (ROC) curve and confusion matrix for the random forest model using the eight selected metabolites (shown in navy) with mean decrease accuracy > 2. Biplot drawn from the microbe- metabolite vectors (mmvec) conditional probabilities estimated for the dataset of **B** CON and **C** PEO groups. Axes correspond to principal components from the singular value decomposition of the microbe-metabolite conditional probabilities estimated using mmvec. Microbes are represented by arrows and metabolites by dots. Heatmaps display the inferred conditional probabilities for various metabolites given the presence of specific microbial taxa in the rumen of goats under **B** CON and **C** PEO groups. Colors indicated protozoal (green) and fungal (pink) genera.

Supplementary Files

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