An integrative metatranscriptomic analysis reveals differences in enteric methanogenesis mechanisms between cows and goats

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Abstract

Background: Reducing enteric methane emissions from farmed ruminants can be achieved by various nutritional strategies. However, it remains unclear to what extent the effects of diet on rumen microbiome are comparable between different ruminant species. In this work, we compared the effects of starch- and/or lipids-rich diets on the rumen microbiome of cows and goats to enhance our understanding of microbial mechanisms of methanogenesis. The study enrolled four Holstein cows and four Alpine goats, conducted simultaneously in a replicate 4×4 Latin square design, receiving the same ration based on grassland hay and concentrate (CTL) or supplemented with corn oil and wheat starch (COS), marine algae powder (MAP) or hydrogenated palm oil (HPO). The microbiome was studied using non-targeted total RNA sequencing.

Results: To identify biologically relevant features, we developed a five-step biostatistical pipeline, combining a network-based approach with clustering and supervised model fitting to associate differentially expressed genes (deKEGGs) and Operational Taxonomic Units (deOTUs) with methane emissions. The COS diet induced the highest methane emissions reduction for cows and goats and most profoundly affected the microbiome. With a focus on the COS *vs*. CTL comparison, the number of deOTUs and deKEGGs in cows (16 and 381) was higher than in goats (10 and 133). Moreover, clustering analysis revealed network topology and functionality differences between ruminant species. In goats, the reduction in methane emissions was strongly associated with genes involved in Carbohydrate metabolism, and genes coding for methylotrophic and hydrogenotrophic pathways of methanogenesis were overrepresented; in cows, only the hydrogenotrophic pathway was prevalent. Further, sPLS analysis identified potential biomarkers characteristic of each ruminant species, such as tetrahydromethanopterin S-methyltransferase and fructose bisphosphate aldolase for cows, and Methanol: coenzyme M methyltransferase, F420-non-reducing hydrogenase for goats.

Conclusions:

Overall, these results demonstrated a strong influence of the ruminant species on the responses of the rumen microbial community to dietary changes. We observed that the COS diet reduced similarly enteric methane emissions in cows and goats, but the induced shifts in the rumen microbiome were not the same. These results suggest that the host-animal species conditions microbial interactions within the rumen ecosystem.

Background

Thanks to a complex microbiota residing in the ruminal compartment, ruminants have the remarkable ability to convert plant biomass that is indigestible for humans into nutrient-dense foods. However, ruminant production is under strong societal pressure due to its high carbon footprint associated with the emission of methane, a potent greenhouse gas (GHG) [1]. For milk production, cows are the most important source of enteric methane, but dairy goats, sheep, and buffalo also contribute [2]. For the last

twenty years, milk production from goats has increased by 2.7% per year, a higher rate than cow's milk [3, 4]. In addition, goats are particularly suited to harsh climatic conditions [4] and linked to climate changes, their numbers and production areas are likely to expand, replacing other livestock species.

Methane production in ruminants results from the activity of methanogenic archaea [5]. It is a natural process linked to the anaerobic hydrolysis and fermentation of feeds, which provides the archaea with the end products of fermentation used for methanogenesis. Rumen methanogens are essentially hydrogenotrophs, utilizing H_2 to convert CO_2 to methane, but other pathways such as methylotrophy are also important [6]. Nutritional strategies appear to be an effective way to mitigate methane emissions from ruminants [7] that farmers could easily adopt without altering the efficiency of animals and other GHG emissions. Several studies comparing diets inducing high and low enteric methane emissions reported changes in ruminal microbial communities, taxa or genes [8-11]. However, no common biomarkers were highlighted, and, in some cases, the results were contradictory.

Notwithstanding, all these studies concur that changes in methane emissions are better reflected by changes in functional gene expression than by changes in microbial populations. Hence, microbial genes may be a more robust benchmark for predicting enteric CH_4 emissions. It is noted that most studies were done on cattle, and there is scarce information on goats. There are some differences in the rumen microbiota of cattle and goats, particularly for bacterial communities [12]. However, it is unknown whether these differences could influence methane production as there are no studies in which these two ruminant species were compared under the same conditions.

A recent study explored the influence of lipid-supplemented diets on methane emissions, feeding behavior and milk fat content in cows and goats [13]. The setup of these simultaneous studies, where diet and management conditions were the same for both ruminant species, provides the opportunity to assess microbial changes associated with methane-reducing diets. We hypothesized that due to anatomical and physiological characteristics and feeding behavior, the rumen microbiome of cows and goats have noticeable differences and that these differences could affect methanogenesis mechanisms. We used metatranscriptomic data and adopted a network-based approach combined with clustering and supervised model fitting to investigate the effects of high starch and lipid supplemented diets on rumen microbial community structure and activity. Both in cows and goats, we searched for diet-specific changes in microbial taxonomical composition and functions related to fermentation patterns and methane-emission phenotypes.

Results

Trials with cows and goats were conducted simultaneously in a 4 x 4 Latin square design (Fig. 1a). Methane emissions were measured in respiratory chambers and rumen contents were sampled before the morning feeding. To study the rumen microbiome, total RNA was extracted from rumen contents and submitted to the RNASeq sequencing approach and further analyzed using a five-step statistical pipeline (Fig. 1b).

Changes in the rumen microbiome match the methane-emitting phenotype.

There were 5,235 KEGGs, and 369 OTUs initially detected after functional and taxonomic mapping in cows and goats. Most of the identified KEGGs were related to Carbohydrate metabolism (11% and 13% in cows and goats, respectively), Genetic information processing protein families (5% and 6.5%), unclassified metabolism functions (4% and 5%), Signaling and cellular processes protein families (4.3% and 4.4%) and Translation (4% and 5%) (**Supplementary Fig. 1a**). Major microbial phyla were presented by Firmicutes (45% in cows and 54% in goats) and Bacteroidetes (24% in cows and 22% in goats), followed by Proteobacteria (8% in cows and 5% in goats) (**Supplementary Fig. 1b**).

In our previous study, only the COS diet decreased CH_4 yield by an average of 28% in both species [13]. The statistical pipeline was run for all dietary comparisons, i.e. COS vs CTL, HPO vs CTL, MAP vs CTL, COS vs HPO, COS vs MAP and HPO vs MAP. Significant changes in the rumen microbiome were found only for COS vs other diet comparisons. Consequently, we present below the comparison of COS with other diets, particularly CTL.

Cows' and goats' microbiomes are similar but respond differently to the diet

In order to make a meaningful comparison, we first examined the microbial structure (in terms of detected 16S rRNA copies) and activity (in terms of detected KEGGs) in cows and goats from the control groups only. The OTUs PCA analysis (**Supplementary Fig. 2a)** showed samples grouping in clusters as a function of the animal species; however, at the phylum level, there was no significant difference in relative abundances, except for *Fibrobacteres*, *Euryarchaeota* and some low abundant phyla like *WPS.2, SR1, Synergistetes* and *Tenericutes*. In a differential expression analysis, we identified only 16 OTUs with significantly different abundance between CTL cows and goats. Two of these OTUs were classified as methanogens, *Methanobrevibacter* and *VadinCA11* (a genus from the *Methanomassiliicoccales* order) and were more abundant in goats. Inversely, an OTU affiliated to *Fibrobacter succinogenes* represented 0.3% of total 16S rRNA counts in CTL cows and less than 0.1% in goats. Similarly, two *Sharpea*-related OTUs were more abundant in cows, while two *Oscillospiraceae* and one *Veillonellaceae* presented higher abundance in goats. The other significantly abundant OTUs represented less than 0.001% of the total counts.

Principal component analysis with the KEGG orthologs showed no clear separation between cows and goats receiving CTL diet (**Supplementary Fig. 2b**). However, out of the 5,235 identified KEGGs, we enumerated 400 that were differentially expressed between CTL animals. Most of them (14%) were related to Carbohydrate metabolism, 9.7% to Signaling and cellular processes protein families, 7% to unclassified: metabolism, 6% to genetic information processing protein families and 6% to Amino acid metabolism. Among these 400 deKEGGs, only a handful had relative abundances higher than 0.01% (7 in cows and 6 in goats). This analysis suggests that despite some differences in rumen microbial

community structure, the microbial activity was similar between goats and cows fed the same CTL diet and under the same simultaneous experimental conditions.

In cows, individual pair-wise comparisons between COS and other diets resulted in 16 to 28 deOTUs, with 10 of them shared in all comparisons (Fig. 2a **and Supplementary Table 1a)**. These 10 deOTUs represent ~ 3% of all OTUs identified. It is worth mentioning that *Succinivibrionaceae*, *Roseburia*, unclassified *RF32*, *Prevotella ruminicola* related OTUs were higher for COS compared to the rest of the diets while the abundance of *VadinCA11*, *Spirochaetaceae*, unclassified *RFP12*, unclassified *Rickettsiales* and *Endomicrobia* OTU was reduced. The pair-wise comparisons between COS and the other experimental diets identified 380 to 791 deKEGGs (Fig. 2b). For each of these deKEGGs and every dietary comparison, the fold change variations in gene expression were always in the same up or down direction. There were 255 shared deKEGGs between dietary comparisons, and they were related to Energy Metabolism (12.5%), Carbohydrate metabolism (12.5%), Genetic information processing protein families (9.8%) and Translation (9%) (**Supplementary Table 1b**). Twenty-five of the 255 COS-specific deKEGGs were discovered to be differently regulated in the CTL diets between species, however, most of these were low abundant (< 0.01%) (Fig. 2e**)**.

In goats, the number of deOTUs and deKEGGs shared across the three pair-wise diet comparisons was lower than for cows. There were four deOTUs (*R4.45B*, *uncl_Rickettsiales*, *SHD.231*, and *Paludibacter* representing 0.61% of all OTUs identified) (Fig. 2c) and 22 deKEGGs (Fig. 2d) (**Supplementary Table 1c and 1d**). Changes in the expression of these 22 deKEGGs in the different diet comparisons were always in the same direction as observed for cows. These 22 shared deKEGGs were related to Unclassified Metabolism (27%), Carbohydrate metabolism (22%), Metabolism protein families (13%), Amino acid metabolism (9%) and Energy metabolism (9%) functions. Twelve of the 22 COS-specific deKEGGs discovered in goats were also found in cows, with just two of them being differently expressed and low abundant (< 0.01%) in CTL diet between species (Fig. 2e).

For each ruminant species, co-occurrence networks were created to analyze the linkages between deKEGGs, deOTUs and other parameters, including VFA, methane, rumen protozoa counts and intake. After clustering using the K-means function, clusters grouping methane metabolism-related variables were used in a pathway enrichment analysis.

For all dietary comparisons in cows, the networks formed 11 clusters (Fig. 3a **and Supplementary Figs. 3a and 3b**). Specifically, the COS vs CTL network had 10,902 edges, 4 upregulated deOTUs (including *Succinivibrionaceae*, unclassified *RF32* and *Prevotella_ruminicola*), 2 downregulated deOTUs (including *Spirochaetaceae* and unclassified *RFP12*), 159 downregulated deKEGGs, 125 upregulated deKEGGs (206 of the network deKEGGs were COS-specific) and 28 parameters associated to VFA composition and protozoa counts among others (**Supplementary Table 2a** for COS vs CTL and **2b** for COS vs MAP and **2c** for COS vs HPO). For each dietary comparison, the two largest clusters of the network contained methane-related variables, some fermentation parameters and deKEGGs related to carbohydrate and energy metabolism. It is noted that daily methane production (CH_4 g/d) is always segregated separately

from CH_4 yield and fermentation parameters. More than one-third of the deKEGGs clustering with methane variables were common between dietary comparisons (75% in COS vs CTL, 36% in COS vs MAP, and 37% in COS vs HPO).

In COS vs CTL we identified clusters 1 and 5 as closely related to methane pathway (Fig. 3a). Cluster 5 included CH_4 yield, the majority of fermentation variables, protozoa counts, an OTU affiliated as unclassified *Spirochaetaceae*, and 95 deKEGGs, most of which related to Metabolic pathways and Microbial metabolism in diverse environments. However, only 3% of the deKEGGs in this cluster were directly connected to the methane metabolism pathway (**Supplementary Fig. 4a**). In contrast, deKEGGs related to the methane metabolism pathway accounted for 16% of all deKEGGs in cluster 1, just after metabolic pathways (26%) and microbial metabolism (20%) (**Supplementary Fig. 4b**). These deKEGGs were identified as subunits of methyl-coenzyme M reductase, tetrahydromethanopterin S-methyltransferase or formylmethanofuran dehydrogenase.

The deKEGGs of clusters 1, 5, and 7 (that contain methane variables) and their linkages with pathways and metabolic reactions were used to create a network of metabolic pathways in cows (**Supplementary Fig. 5a**). Some pathways had numerous interactions with the deKEGGs networks, and these corresponded to methane metabolism, secondary metabolite biosynthesis, carbon metabolism, microbial metabolism, metabolic pathways; deKEGGs from cluster 1 interacted primarily with the pathways with a high degree of interactions, such as the methane metabolism.

COS vs HPO and COS vs MAP network cluster distributions were comparable to COS vs CTL, although *Methanosphaera* was in one of the biggest clusters with CH_4 yield and *Methanobrevibacter* clustered with CH_4 g/d in COS vs HPO (**Supplementary Fig. 3a** for COS vs MAP and **3b** for COS vs HPO).

The same analytical approach in goats allowed the construction of a co-occurrence network in COS vs CTL of 518 edges containing 22 fermentation parameters, 2 downregulated deOTUs (uncl_BS11 and *Pirellulaceae*), 26 downregulated deKEGGs, and 37 upregulated deKEGGs (Fig. 3b). The co-occurrence networks for COS vs MAP and COS vs HPO included only a few microbial variables and were not subjected to further analysis (**Supplementary Fig. 3c** and **Supplementary Table 3a** for COS vs CTL **and 3b** for COS vs MAP).

Clustering of the COS vs CTL network in goats resulted in 5 clusters, two of them (cluster 1 and cluster 5) related to methane metabolism. It is noted that daily methane production is segregated in the same cluster with CH_4 yield and fermentation parameters (cluster 5). **Supplementary Fig. 4c** shows the pathway analysis of cluster 5, which revealed that Metabolic pathways, Microbial metabolism in diverse environments, Methane metabolism (8 deKEGGs), and Carbon metabolism accounted for 85% of deKEGGs and in less proportion with Signal transduction and Translation. The pathway analysis of cluster 1 from goats revealed similar pathways to those found in cluster 5, as well as glycolysis/gluconeogenesis, ABC transporter, Fructose and mannose metabolism, and Glutathione metabolism (**Supplementary Fig. 4d**).

Pathways with a high degree (more interactions) were also found in the goat integration network (**Supplementary Fig. 5b**), such as metabolic pathways, microbial metabolism, carbon metabolism, and methane metabolism, which were mostly associated with a group of deKEGGs belonging to cluster 1 and 5 of the co-occurrence network. Sulfur metabolism, purine metabolism, galactose metabolism, porphyrin metabolism, and pentose phosphate patwhays were linked with glycolysis/gluconeogenesis and connected with deKEGGs from cluster 1.

Potential biomarker genes were identified in cows and goats.

In a way to identify a set of variables that maximize the co-variation between the host-methane emissions and the microbial-derived traits (deOTUs, deKEGGs), the final stage in the pipeline implemented an sPLS regression analysis on the data from the previous clustering selection. **Supplementary Tables 4 and 5** for cows and goats, respectively, include the final results of the pipeline. In cows, our analysis highlighted 25 discriminant variables (Fig. 4a) from cluster 1; eight were genes involved in the methanogenesis pathway. In goats, 30 of the variables in cluster 5 were shown to be discriminant (Fig. 4b); and eight were methanogenesis-related genes. Only three discriminant deKEGGs were shared between the two animal species (**Table 1**). Moreover, all discriminant deKEGGs in cows and most of them in goats were downregulated in differential expression analysis and with positive loadings in the PLS analysis with the COS diet, indicating a negative relationship with methane emission (which decreased when COS was fed to animals).

In cows, the sPLS analysis to forecast CH_4 (g/d) variation in each cluster showed that cluster 1 had the greatest prediction ability (58.7%), and clusters 5, 7 and 9 explained 45% of the variability. In goats, cluster 1 explained the variability of CH_4 (g/d) by 84%, while cluster 5 by 40%.

Discussion

Diet and feed additives are the most effective strategies to reduce enteric methane ruminant emissions which could be easily, and rapidly applied in ruminant husbandry. Feeding lipids in combination with starch successfully decreases methane emissions in cows [14–16], whereas data on goats are scarce and inconsistent [17, 18].

In a previous work [13], we reported that the COS diet, supplemented with corn oil and wheat starch, decreased CH_4 yield in the same proportion in cows and goats compared to CTL, whereas HPO and MAP diets supplemented respectively with hydrogenated palm oil or marine algae powder had no effect on methane emissions.

These comparable results in both species offered an excellent opportunity to investigate microbial mechanisms of methane mitigation strategies in cows and goats. We hypothesized that the COS diet similarly affected microbial structure and functions in both species, as the methane production was uniformly reduced. Therefore, we built a five-step statistical pipeline to analyze the metatranscriptomic data from rumen samples of cows and goats by integrating analogies and discrepancies of microbial

responses to a diet aimed at uncovering finely-tuned mechanisms of methanogenesis in cows and goats. With this objective, we focused on a detailed examination of the outputs of each pipeline step before comparing the final step outputs in COS vs CTL, COS vs HPO and COS vs MAP comparisons. The final goal was to identify biomarkers that describe major methanogenic pathways in the rumen of these ruminant species.

Diet is considered as the main factor affecting rumen microbiota, though the abundance of microbial groups varies between ruminant species [12]. In their extensive-scale survey, Henderson et al. [12] highlighted that *Veillonellaceae* and *Fibrobacter* guided the clustering of bovines and caprids. In accordance with this work, we also observed a higher abundance of *Fibrobacter* and a lower abundance of *Veillonellaceae* in cows compared to goats when receiving the control diet. Though there were some differences in microbial community structure, the feed degradation and fermentation pathways seem complementary, as we did not find any major KEGGs differentially expressed between cows and goats. This complies with CH_4 yield results, which were highly similar between cows and goats receiving a control diet.

In contrast, changes in methane emissions due to diet were better correlated with gene expression than with microbial taxonomy, as the number of differentially deKEGGs was higher than that of deOTUs across diets in both species. A change in the rumen microbiota composition could have been expected as a consequence of the lipids supplemented in the diet; toxic effects were previously reported in pure cultures [19] and *in vitro* [20] and to some extent *in vivo* [21]. There was no change in microbial gene expression when cows and goats were receiving the HPO and MAP diets, compared to the control. As discussed in our companion paper [13], the lipids supplementation level applied with these diets was low (up to 3% of DMI), and supplementing fats up to 6% had shown no adverse effects on total nutrient digestibility and total VFAs [22] with probably limited detrimental effects on rumen microbes. The COS diet was the only that affcted microbial gene expression, demonstrating a shift in microbial metabolism. This diet's effect can be attributed to higher starch and lipid supply.

The COS diet considerably modified microbial gene expression in cows and to a much lower extent in goats, as attested by the number of deKEGGs shared between all dietary comparisons and identified for each species, i.e. 255 for cows and 22 for goats. Nevertheless, in both species, carbohydrate metabolism was one of the primary categories affected by the diet indicating a switch in fermentation pathways.

Correlations between deKEGGs, deOTUs, and fermentation parameters, including individual methane measures and protozoa counts, were used to construct a compositionally-corrected network; this step, combined with the k-means clustering, assured the selection of biologically relevant microbial correlation data. A network was depicted for each dietary comparison in cows, while for goats, we used only the COS vs CTL network (although smaller than those in cows) as COS vs MAP and COS vs HPO comparisons resulted in insignificant correlations between variables. The disparity between cows and goats here could be explained by the time of sampling and behavioural patterns. Rumen content samples were taken before the morning feeding when rapidly fermentescible organic matter is absent; in this context,

identified deKEGGS indicate sustained and long-lasting changes in microbial gene expression. On the other hand, we observed that goats ate faster and more frequently and had a higher ruminal turnover rate than cows [13]. Therefore, we could hypothesize that this shorter rumen retention time prevented the lipids from persistently modifying microbial activity.

A common characteristic between cows and goats is the clustering of CH_4 yield predominantly with genes involved in various metabolic pathways and excluding most of the methane pathway genes, which formed another cluster in the networks. Taking the analysis a step further, we identified the processes involved in the methane variability for each identified cluster. In goats, the variability of methane emissions was explained primarily by carbohydrate metabolism processes. In contrast, in cows, this variability was explained by carbohydrate and methane metabolism clusters. These results suggest that the amount of produced methane is closely related to the activity of bacteria providing substrates for methanogenic archaea. This is congruent with research that showed that variances in methane in cattle are primarily explained by other microbial communities and their activities rather than being driven only by methanogens [9, 23].

Our analysis revealed that the enzymes tetrahydromethanopterin S-methyltransferase (Mtr) and fructose bisphosphate aldolase (Fba) characterized the relationship between cows microbiome and methane emission, whereas Methanol:coenzyme M methyltransferase (Mta), F420-non-reducing hydrogenase (Mvh) were highlighted in goats. The 5,10-methylenetetrahydromethanopterin reductase (Mer), methyl-coenzyme M reductase (Mcr), and formylmethanofuran dehydrogenases (Fwd) enzymes were found in both species; all of these were downregulated by COS supplementation.

Likewise, another point of difference was the enzyme Mta involved in the methylotrophic pathway was specific to goats receiving the COS diet. Methylotrophs have a lower H_2 threshold and a thermodynamic advantage in that they are driven by the availability of methyl compounds rather than the concentration of dissolved H_2 [24]. It was also found that corn oil and fish oil supplementation lowered methyl-compounds plasma availability [25]. These findings indicated that goats were more responsive to alterations in methylotrophic pathway supplies, and this inhibition is critical for lowering CH_4 expression since MAP modulated some genes on methanogenesis but not this pathway.

This exploratory study provides useful data explaining the differences in microbial mechanisms involved in methane production between cows and goats.

Conclusions

To our knowledge, this is the first direct comparison of cows' and goats' microbiomes integrating methane emissions. Moreover, the rumen microbial ecosystem was only poorly characterized in goats before. Our results showed that in both ruminant species, CH_4 yield clustered closely with genes involved in carbohydrate metabolism, which highlights the importance of diet and the production of substrates available for methanogenesis rather than the structure and activity of the methanogens population.

Besides, genes from the methanogenesis pathway identified as biomarkers were different in cows and goats, though changes in methane emissions were similar. These findings suggest that the establishment of microbial interactions in the rumen depends on the host-animal physiology and, in particular, the feeding behaviour.

Methods

1. Experimental methods

The Auvergne Rhône-Alpes Ethics Committee approved the experimental protocol for Animal Experiments (France; DGRI agreement APAFIS#3277–2015121411432527 v5), which conformed with European Union Directive 2010/63/EU norms. Experiments were carried out at the U.E. HerbiPole LowMountain Ruminant Farming Systems Facility, https://doi.org/10.15454/1.5572318050509348E12 at Theix, 63122 Saint Genès Champanelle from February to July 2016. The experimental protocols are depicted in Fig. 1a.

1.1. Diets, animals, and experimental design.

Lactating and multiparous Holstein cows (n = 4) and Alpine goats (n = 4) were enrolled in a 4 x 4 Latin square design as previously described (Fougère et al., 2018). Briefly, animals were randomly assigned to one of four experimental diets: i) CTL grassland hay and concentrate in a 45:55 ratio on a dry matter (DM) basis containing no additional lipid, ii) COS, which was a CTL diet supplemented with corn oil (5.0% of total dry matter intake (DMI)) (Olvea, Saint Léonard, France) and wheat starch, iii) MAP, CTL + 1.5% DMI of *Schizochytrium sp.* marine algae powder (DSM, Basel, Switzerland), or iv) HPO, CTL + 3% DMI of hydrogenated palm oil (Provimi, Cargill, Saint-Germain-en-Laye, France). Dietary formulations are described in Fougère et al., 2018 and [13] and summarized in **Supplementary Table 6.** Each experimental period lasted 28 days, and at the beginning of each period, animals were adapted to the diet for 6 days. Experimental diets were fed ad libitum twice daily (08h30 and 16h00) except from day 21 to 27, when animals were moved to open-circuit respiration chambers and received 95% of individual voluntary feed intake to ensure complete feed consumption. The animals had access to a constant supply of fresh water ad libitum.

1.2. Measurements, sampling, and chemical analysis.

1.2.1. Enteric methane emissions.

Enteric methane emissions measures were described in [13]. Methane emissions (g/day) expressed per unit of intake corresponded to CH_4 yield (g/kg dry matter intake (DMI)) and per unit of milk to CH_4 intensity (g/kg milk).

1.2.2. Ruminal fermentation parameters.

Ruminal fluid samples (500 mL) were collected by stomach tubing [13] before the morning feeding on day 27 of each experimental period when animals quitted the respiration chambers. Samples were subsampled for volatile fatty acid (VFA), ammonia (NH_3) [26] and protozoa analyses [27]. Protozoa were

counted by microscopy, log-transformed, and classified as small *Entodiniomorphs* (< 100 µm) (small_Ento) or large Entodiniomorphs (> 100 µm) (large_Ento), or as *Holotrichs* (*Dasytricha (Dasy)* or *Isotricha (Iso))* [28].

1.2.3. Rumen microbial RNA extraction and sequencing.

RNA from rumen liquid fraction was extracted using RNeasy Plus Mini Kit, as previously described [29]. Total extracted RNA was sent to Centre d'expertise et de services Génome Québec for Illumina NovaSeq 6000 for paired-end sequencing. Raw data are available in the link at the data and material section availability.

2. Bioinformatic analysis.

Shotgun metatranscriptomics of 32 rumen content samples obtained from Holstein cows and Alpine goats were analyzed using the MetaTrans open-source pipeline [30]. Using the FastQC tool [31] and the Kraken pipeline [32], the raw paired-end readings were subjected to quality control, such as the per base N content (< 5%), the read length (minimum 150 bp) and the per sequence quality score (mean quality > 27). The SortMeRNA tool [33] was used to separate rRNA/tRNA data from non-rRNA/tRNA reads, the latter being potential mRNAs. The reads classified as rRNA/tRNA were taxonomically annotated against the 16S rRNA gene Silva v138.1 database using SOAP2 [34]. Prior to that, the two single reads from the DNA fragment were overlapped using Fastq-Join [35], and these sequences were clustered using the UCLUST method [36]. As a result, ribosomal RNA reads were grouped in Operational Taxonomic Units (OTUs) at 99% sequence similarity. For functional annotations, the non-rRNA/tRNA reads were mapped to a catalogue of microbial genes from the bovine rumen [37] using the SOAP2 tool after being subjected to the FragGeneScan tool [38] to predict potential genes for the functional analysis and predicted genes being subjected to clustering using CD-HIT v4.6 [39] an identity threshold of > = 95% and gene overlap of > = 90%. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and the evolutionary genealogy of genes of non-supervised orthologous groups (eggNOG) databases were used to annotate the database. The bioinformatic analysis metrics are shown in **Supplementary Table 7**.

3. Biostatistical analysis.

The pipeline was built using the R programming language. The R script and RData file are available at Code availability. The statistical workflow is depicted in Fig. 1b, which includes the sequential steps of differential expression, co-occurrence, clustering, pathway enrichment analysis, and supervised statistical approaches (sPLS). Pair-wise comparisons of all diets (COS vs CTL, COS vs MAP, COS vs HPO and CTL cows vs CTL goats) were performed to investigate diet-dependent changes in the abundance of KEGGs and OTUs within each species.

In the *vegan* R library [40], *anosim* function was used to determine whether the median of two or more groups of samples were statistically different, and *betadisper* function was used to check OTUs and KEGGs dispersion between experimental groups and construct the Principal Component Analysis (PCA) figure.

3.1. Differential expression analysis.

DESeq2 R package [41] with the Wald test were used to make differential expression pair-wise comparisons among diets. To control the false discovery rate (FDR), the Benjamini–Hochberg approach [42] was used to compensate for multiple testing. KEGGs and OTUs with an FDR-adjusted $P < 0.05$ were considered differentially expressed and referred to hereafter as deKEGGs and deOTUs (Fig. 1b, **step 1**).

3.2. Co-occurrence networks analysis.

In order to examine bivariate unconditional associations between variables, matrices with deKEGGs and deOTUs from pair-wise comparisons were used to compute co-occurrence networks using the *ccrepe* package in R [43] (Fig. 1b, **step 2**). The fermentation parameters (total volatile fatty acids (VFAs, mmol), acetate (C2), propionate (C3), butyrate (C4), isobutyrate (isoC4), valerate (C5), isovalerate (isoC5), caproate (C6) represented as mmol/mmol of total VFAs, ratios C2/C3, C2 + C4/C3, NH3 (mMol), DMI (kg/d), organic matter intake (OMI ((kg/d)), CH_4 (g/d), CH_4 yield, CH_4/CO_2) but also individual protozoa counts (log-transformed) (total protozoa, small_Ento, large_Ento, Iso, Dasy) were integrated in the deKEGGs and deOTUs input matrices.

The co-occurrence inference graph was created using Pearson correlation [44]. Each input matrix was divided by the sum of its corresponding column for data transformation. The Reboot approach was used to resample the edge scores. Edge scores were first randomized using 1000 permutations of row shuffling (for null distributions), followed by 1000 permutations of bootstrapping (for s randomization). In brief, the correlation of measure scores is used to calculate a correction factor and show the associations' strength. A threshold of the p-values of the edges assigned to node pairs and the corrected significance (q-value) was set at $P < 0.05$ and q-value < 0.001. Cytoscape software (cytoscape.org) was employed to visualize the inferred network and cluster (Fig. 1b).

3.3. Clustering analysis.

A clustering algorithm was applied to categorize the co-occurrence networks into functionality (KEGGs) and/or taxonomically (OTUs) enriched units. We applied a K-means clustering approach using the Hartigan and Wong algorithm, implemented in the *stats* package in R (R Core Team, 2019) (Fig. 1b, **step 3**). The matrices of significantly correlating deKEGGs, deOTUs and fermentation parameters were used as inputs in this analysis.

3.4. Pathway analysis.

The *KEGGREST* package [45] was used to explore the biological relevance of network clusters. The list of deKEGGs of each cluster was used to find enrichment in diverse biological categories; KEGG pathway mapping was used to link the KO annotation (K number assignment) to the KEGG pathway maps (https://www.genome.jp/kegg/mapper/reconstruct.html). The deKEGGs of co-occurrence network clusters and their linkages with pathways and metabolic reactions were used to create a network of metabolic pathways; the pathways were then categorized by degree of interactions, and the deKEGGs by associated pathway (Fig. 1b, **step 4**).

The *KEGGREST* package was also used to investigate reactions and metabolites related to deKEGGs of each cluster. Cytoscape was used to visualization of results.

3.5. Multivariate analysis.

The clusters most closely related to the methane metabolism pathway (map00680) were subjected to multiple linear regression analysis to examine the joint associations of the selected cluster variables (matrix X) with different Y matrices: i) CH_4 (g/d), ii) CH_4 yield, in order to look at conditional associations. Sparse Partial least Square (sPLS), as implemented in the *mixomics* r package [46], was used to perform the multivariate analysis (Fig. 1b, **step 5**). The *perf* and *tune* functions were used to select the model's number of components and variables. Mfold validation, Mean Absolute Value (MAE) as a measure, folds = 5 and the number of repetitions = 10 were used in the validation process. Subsequently, we extracted the 25 variables that most affected each Y variable, with the highest absolute loading value.

Abbreviations

Small_Ento, small *Entodiniomorphs*

Large_Ento, large *Entodiniomorphs*

Iso, *Isotricha*

Dasy, *Dasytricha*

CH_4 , methane

CO_2 , carbon dioxide

H_2 , hydrogen

Mtr, tetrahydromethanopterin S-methyltransferase

Fba, fructose bisphosphate aldolase

Mta, Methanol:coenzyme M methyltransferase

Mvh, F420-non-reducing hydrogenase

Mer, methylenetetrahydromethanopterin reductase

Mcr, methyl-coenzyme M reductase

Fwd, formylmethanofuran dehydrogenase

Fbp, Fructose 1,6-bisphosphatase

CTL, control diet

COS, control diet + corn oil and wheat starch supplementation

MAP, control diet + marine algae powder supplementation

HPO, control diet + hydrogenated palm oil supplementation

VFA, Volatile Fatty Acids

FMD, Fat Milk Depression

NH_3, Ammonia

DMI, Dry matter intake

OMI, Organic matter intake

deOTU, Operational taxonomic unit differentially expressed

deKEGGs, The Kyoto Encyclopedia of Genes and Genomes (genes) differentially expressed

sPLS, Sparse partial least square

FDR, False discovery rate

C2, Acetate

C3, propionate

C4, butyrate

isoC4, isobutyrate

C5, valerate

isoC5, isovalerate

C6, caproate

Declarations

Ethics approval and consent to participate

The Auvergne Rhône-Alpes Ethics Committee approved the experimental protocols for Animal Experiments (France; DGRI agreement APAFIS#3277–2015121411432527 v5), which conformed with European Union Directive 2010/63/EU norms.

Consent for publication

Not applicable

Availability of data and material

Data availability: http://www.ncbi.nlm.nih.gov/bioproject/762012

Code availability: https://github.com/kfcorral/Cows-and-Goats-Microbiota

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Corral-Jara Karla Fabiola: Metatranscriptomic formal analysis, Conceptualisation, Writing - original draft. **Ramayo-Caldas Yuliaxis:** Metatranscriptomic formal analysis, review & editing. **Bernard Laurence:** Funding acquisition, Conceptualization, Data curation, review & editing. **Martin Cécile:** Funding acquisition, Conceptualization, Data curation, review & editing. **Tournayre Jeremy:** Bioinformatic support, review & editing. **Morgavi Diego:** Funding acquisition, Conceptualisation, Supervision, Writing - review & editing. **Popova Milka:** Funding acquisition, Conceptualisation, Supervision, Writing - review & editing.

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Table

Table 1 is available in the Supplementary Files section

Figures

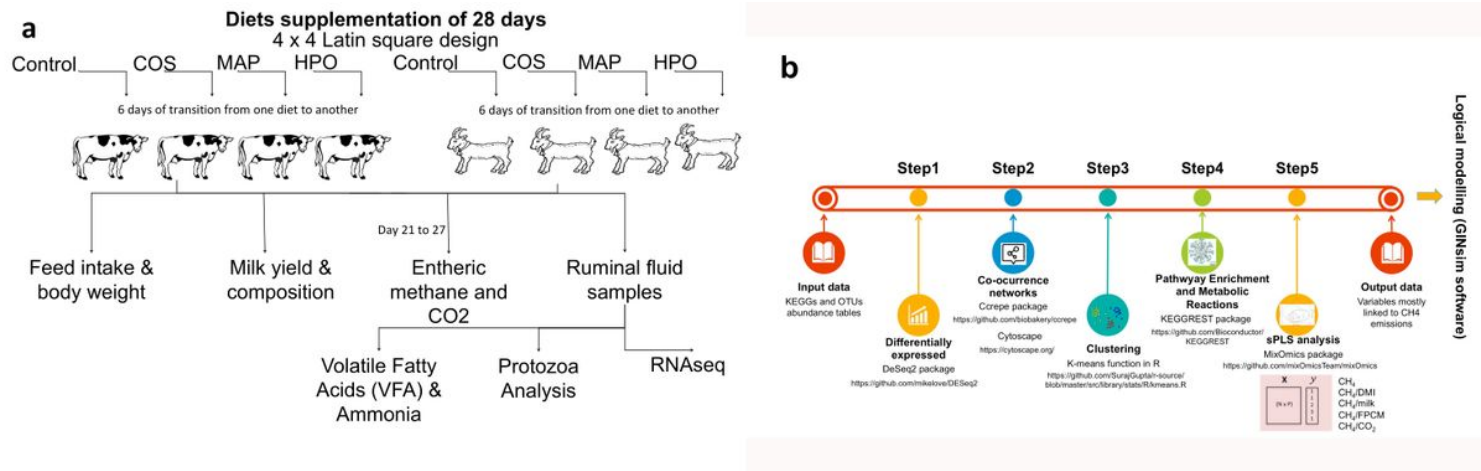

Figure 1

Methodological frameworks. a) The experimental strategy implemented. Cows were fed a control (CTL) and three experimental diets COS (CTL + corn oil and wheat starch), MAP (CTL + marine algae powder and HPO (CTL+ hydrogenated palm oil) over 28 days period. Methane emissions were measured in respiratory chambers from day 21 to 27, and rumen fluid for microbial and fermentation patterns analysis were sampled on day 28. **b)** Statistical workflow for RNA-seq data analysis. Tables of abundance for gene transcripts and OTUs underwent a differential expression analysis using the DeSeq2 package; the

Ccrepe package was further used to establish co-occurrence networks visualized in Cytoscape. After the clustering step, biologically relevant clusters were subjected to pathway enrichment. Potential microbial markers were identified after an sPLS analysis using the mixOmics package.

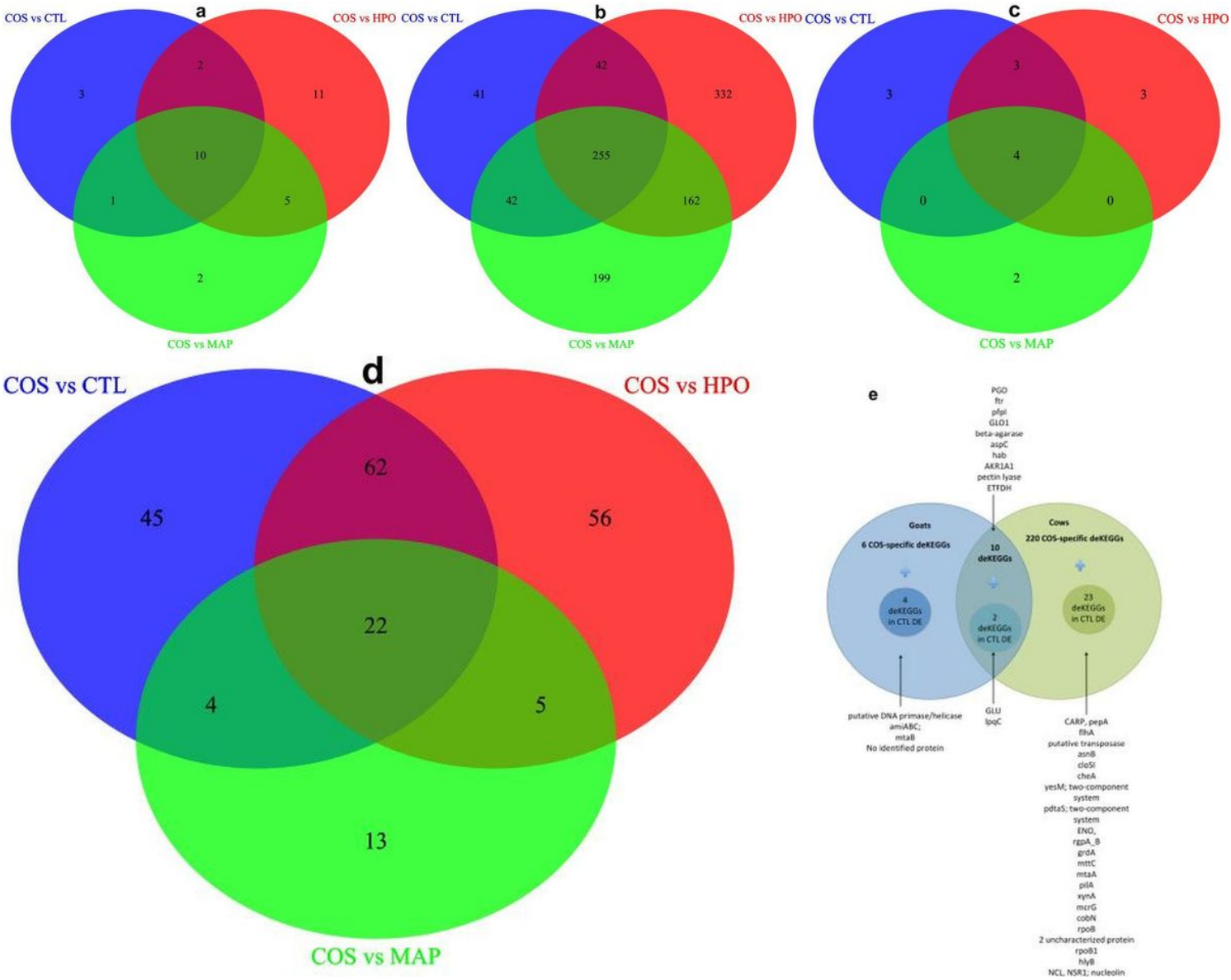

Figure 2

Venn diagrams of a) deOTUs and **b)** deKEGGS in cows and **c)** deOTUSs and **d)** deKEGGs in goats, identified in the three diet comparisons: COS vs CTL, COS vs MAP and COS vs HPO, **e)** Integration of COS-specific deKEGGs in cows and goats.

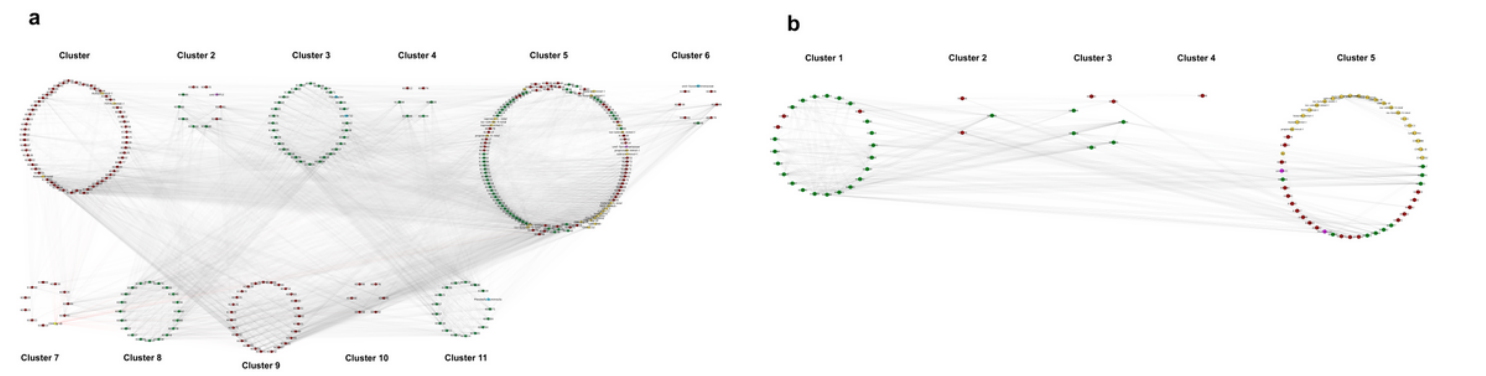

Figure 3

Construction of co-occurrence networks. a) Cow network in COS vs CTL comparison and **b)** goat network in COS vs CTL comparison. Red nodes represent downregulated deKEGGs, green nodes are upregulated deKEGGs, blue nodes are upregulated deOTUs, pink nodes represent downregulated deOTUs and yellow nodes are fermentation parameters.

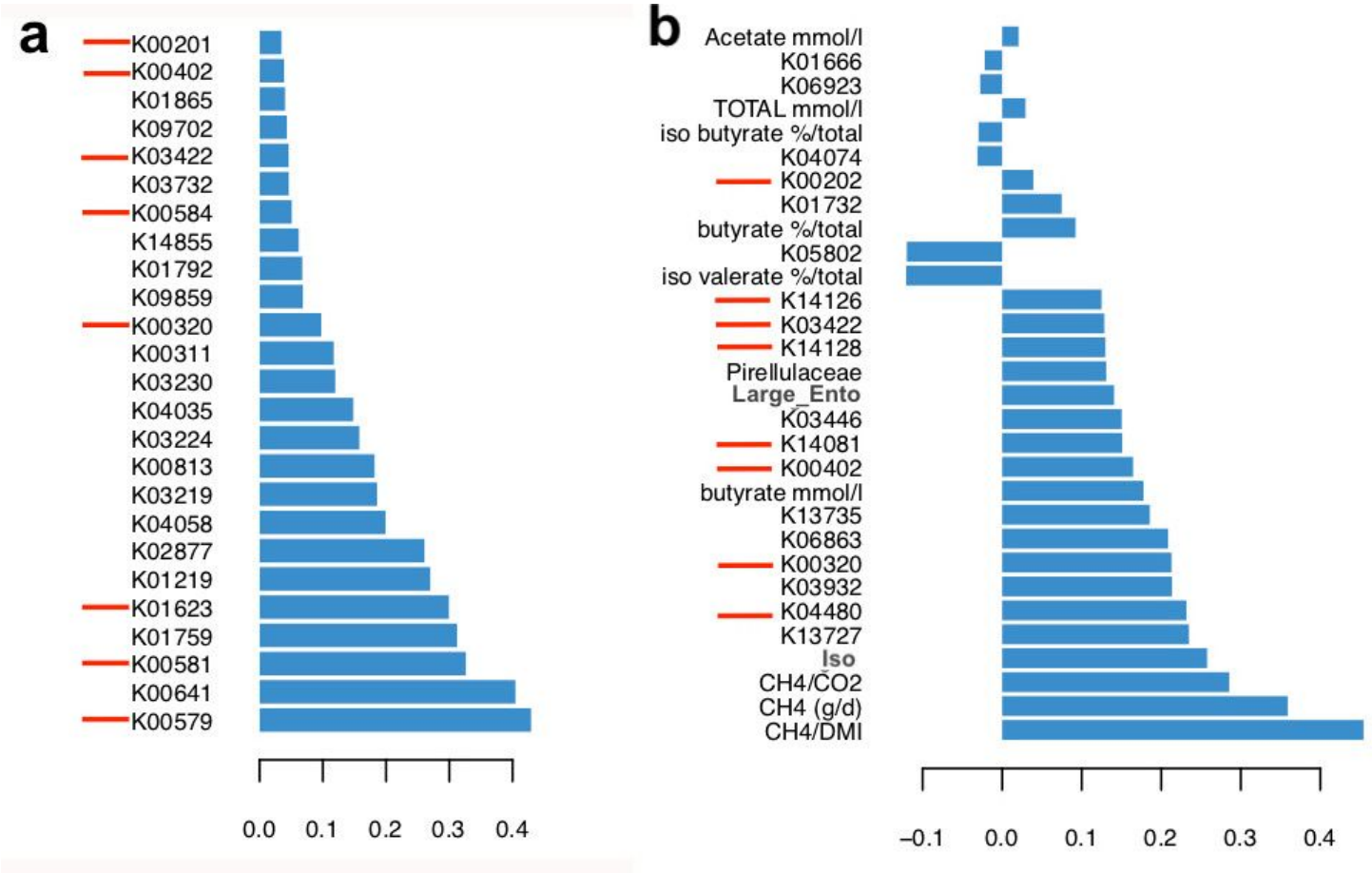

Figure 4

Loading plots of PLS regression analysis in COS vs CTL comparison. a) 25 variables of PLS cluster 1 in cow analysis. **b**) 30 variables of PLS cluster 5 in goat analysis. Red lines represent genes related to

methanogenesis.

Supplementary Files

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