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Paper

# Comparative Analysis of the Methanogen Microbiota Associated to Pasture and Stall Housing in Kazakh Cattle

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## Abstract

The microbial community of cattle rumen (archaea) are the key players in methane emissions. Methane pollutes the atmosphere and leads to the loss of feed energy. The aim of this study was to comparatively investigate the cattle microbiota, with a particular focus on archaea, in relation to the type of housing: pasture versus stall. A 16S metabarcoding analysis of the intestinal contents of cattle was carried out. Alpha - diversity of grazing animals showed to be higher compared to animals in the stall period (p=0.002). Beta - diversity confirmed a difference in methanogens and microbiota between animals kept on pasture and those in a stall. Differential abundance analysis showed that the relative abundance of the *Methanobacteriacea* family in animals in the pasture period was significantly higher compared to animals in the stall period (FDR p = 0.00122). In conclusion, this study demonstrates that the concentration of methanogens in the fecal contents of animals during pasture period was higher than that in animals during the stall period. We recommend feeding grazing animals with concentrates in the evening in order to mitigate methane emissions.

## Keywords

Archea, Cattle, Kazakhstan, Methane, Microbiota

## *Introduction*

Methane ($CH_4$) is one of the main greenhouse gases. It has been estimated that livestock globally contributes between 9 and 11% of total anthropogenic greenhouse gas emissions, of which about 44% of livestock emissions are in the form of methane. The largest source of these emissions is intestinal fermentation in ruminants (Ross et al., 2013). Intestinal methane emissions from ruminants account for approximately 15% of all methane emissions. In this regard, the measurement and reduction of methane emissions from livestock are becoming more important.

The microbial community of cattle rumen includes bacteria, fungi and protozoa. Among these, archaea (methanogens) are key players in methane emissions (Wallace et al., 2019; Matthews et al., 2019).

Archaea are strictly anaerobic and are the only microorganisms present in the rumen capable of producing methane. They are found in the rumen content at a concentration of $10^6$ to $10^8$ cells / ml, costituiting less than 4% of the microbial community. Due to their reliance on the end products of fermentation as substrates, Archaea occupy the bottom of the food chain. According to a meta-analysis of global data, 90% of rumen methanogens belong to the following genera: *Methanobrevibacter* (63.2% of the methanogenic population), *Methanomicrobium* (7.7%), *Methanosphaera* (9.8%), "Rumen Group C", now referred to as *Thermoplasma* (7.4%) and *Methanobacterium*(1.2%). The hydrogenotrophic rumen methanogens primarily belong to the genus *Methanobrevibacter*, whereas the main methylotrophic rumen methanogens belong to the genus *Methanosphaera* and the order *Methanomassiliicoccales*. *Methanobrevibacter* is the most prevalent genus of methane producers in the rumen, accounting for 75-78% of methanogenic archaea (Bach et al., 2018; Kelly et al., 2019).

As a result of digestive processes in the rumen, by-products of fiber breakdown and fermentation end products are formed, including hydrogen ($H_2$), carbon dioxide ($CO_2$), methanol, methylamines, and methylsulfides which are not



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utilized by the host animal. Hydrogenotrophic and methylotrophic methanogens in the rumen are capable of removing these end products, converting them into methane ($CH_4$), which animals expel leading to emissions and atmospheric pollution. Methanogens convert $H_2$ and $CO_2$ into $CH_4$ through a 7-step enzymatic pathway ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$) (Ross et al., 2013; Kelly et al., 2019). This process provides methanogens with an energy source, and also reduces the concentration of $H_2$ in the rumen (Ross et al., 2013). Methanogenic archaea are the primary consumers of $H_2$ in this ecosystem, producing $CH_4$ as the final product (Martinez-Fernandez et al., 2016). Methane synthesis prevents the accumulation of $H_2$ in the rumen, which would otherwise suppress the normal function of microbial enzymes involved in electron transfer. Indeed, if ruminants did not produce methane, the pH of the rumen would fall, hampering the digestion of fiber (Matthews et al., 2019).

The production of methane by the archaea of the rumen leads to a loss of feed energy ranging from 2-12% of the total energy consumed that could be used for production purposes. If methanogenesis was suppressed and available hydrogen [H] was redirected to alternative energy-intensive metabolic pathways, an increase in productivity could be expected (Martinez-Fernandez et al., 2016).

Various perspectives exist regarding the correlation between methane production and feeding efficiency. One hypothesis posits that energy is not lost, as methane generated during digestion can be retained in body weight gain, thereby enhancing overall animal productivity. Being Methane a byproduct of digestion, its levels may be indicative of a more thorough digestive process, ultimately resulting from an increased nutrient availability in the feed (Harvey et al., 2020). In an *in vitro* study employing an experimental model, the authors did not find significant differences in methane production among steers with varying average daily growth (Freetlyet al., 2015). Conversely, other researchers argued that reducing methane production increases the efficiency of feeding, as carbon -rather that methane-, could be utilized for animal growth (Myera, 2019).

Dietary manipulation is considered the most effective and convenient approach to reduce methane emissions (and, thus, the energy loss in animals) and enhance nitrogen utilization efficiency (Matthews et al., 2019). Various strategies, mostly based on dietary modifications, are currently under investigation to reduce methane emissions from ruminants, including improvements in feed quality and feeding systems, (Matthews et al., 2019; Kelly et al., 2019).

Ruminants play a crucial role in the economies of many developed and developing countries. The challenge of mitigating $CH_4$ emissions from ruminants is a global concern for stakeholders. A thorough comprehension of the interplay between microbial activity in the gastrointestinal tract of cattle and methane emissions is essential. A better understanding of this process is pivotal for refining strategic measures aimed at reducing methane emissions, thus striking a delicate balance between sustainable food production and greenhouse gas impact.

Thus, modifying the diet of ruminants in order to reduce the amount of methanogens can contribute to limiting the environmental impact and improving the efficiency of animal husbandry. The dietary composition, in turn, is contingent upon the type of animal management. Currently, the issues of methane emission, depending on method of animal keeping, remains insufficiently elucidated by the international scientific community. In this regard, our study aims to contribute new insights and advance our understating of this critical issue.

Previously, we conducted studies of the microbiota of the gastrointestinal tract of cattle from different regions of Kazakhstan (Daugaliyeva et al., 2022).

The aim of this study was to comparatively investigate the microbiota, with a particular focus on archaea, in cattle, in relation to the type of animal housing.

## Materials and Methods

### Animals

The experiment adhered to the regulations for the treatment of animals (order of the Minister of Agriculture of the Republic of Kazakhstan dated December 30, 2014 No. 16-02 / 701) and received approval by the Bioethics Commission of the Kazakh Research Institute for livestock and Fodder Production. A total of 49 cattle belonging to Kazakh White-Headed, Angus, Holstein and Alatau breeds, aged 3-4 years, were selected for the experiment from the following regions of Kazakhstan: Southern, Western, Northern, Central, Eastern. In total, fecal samples were taken rectally from 22 heads during the stall period, and from 27 heads during the pasture period. Immediately after sampling, faeces were placed in sterile containers, which were delivered to the laboratory in a thermal suitcase with ice packs, and stored at -80 °C (Shoukun et al., 2017).



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## DNA extraction, 16S rRNA library preparation and Sequencing

Total genomic DNA was isolated from 200 mg of faeces per sample using the DNeasy® PowerSoil® Pro Kit (QIAGEN GmbH, Germany) in accordance with the manufacturer's protocol. In addition to the samples, a positive control consisting of a standard microbial community (MOCK, Zymo Research) and a negative extraction control was set up. The negative extraction control consisted of lysis buffer in the absence of any biological material. Genetic libraries were prepared using primers specific to V3- V4 regions in accordance with the manual "Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System" (No. 15044223 rev. A). Library quality was assessed with the Qubit@ 2.0 fluorometer (Invitrogen, Thermo Scientific, CA, United States) and the Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, United States). Molecular-grade water replaced DNA in library controls during PCR amplifications.. Ready-made DNA libraries with the addition of PhiX were sequenced using the MiSeq® Reagent Kit v3 for 600 cycles (Illumina Inc., USA). NGS sequencing of 16S amplicons was performed on a MiSeq Illumina instrument (Illumina Inc., USA).

## Statistical and bioinformatics analysis

Student t-test was used to calculate significant changes in the methanogen Archea abundance between the two housing groups. The 16S metabarcoding data obtained from the samples of intestinal contents were analyzed using the Data QC workflow. The raw FASTQ data underwent analysis using the 'Data QC and OTUs Clustering' and 'Estimate Alpha and Beta Diversity' workflow tools of the Microbial Genomics Module in the CLC Genomic Workbench v. 23.0.2 software (Qiagen). Paired-end reads were joined and trimmed for low-quality score (Qscore < 0.05), nucleotide ambiguity (max of 2 nucleotides allowed), adapter sequences, and length. Duplicate sequences were merged and aligned against the SILVA database (v. 138) at 97% identity threshold. Chimeric reads were removed, and taxonomy was assigned, creating an OTUs table. Profiles of the negative control and the mock communities were examined to assess procedure correctness and rule out cross-contamination, then not consider further. As rarefaction curves did not reach a plateau in all study samples, comparisons were made at a sequencing depth of 60,000 reads. Alpha diversity (diversity within the groups) was estimated using the Chao-1 bias-corrected method, while Weighted UniFrac parameter and Principal Coordinates Analysis (PcoA) were employed to assess beta - diversity (diversity between groups). Statistical support for alpha diversity was determined using the Kruskal–Wallis test, while the PERMANOVA test was applied for beta diversity based on Bray-Curtis, Jaccard, and Euclidean matrices. The OTU table reporting the abundance at the taxonomic level of family was used to perform a generalized linear model test of differential abundance based on housing type. The 'Differential Abundance Analysis' tool in CLC Genomic Workbench performs a TMM normalization to ensure compatibility among samples by adjusting library sizes. Finally, a heat map was generated to explore associations between housing type and microbiota composition, incorporating families for which statically significance was detected following the differential abundance analysis.

# *Results*

The comparative analysis of the archaeal content in the microbiota of the gastrointestinal tract of cattle during pasture and stall periods, showed that the absolute abundance of methanogen Archaea was higher in the faeces of cows in the pasture period than in the stall period (Table I). These differences were found to be highly statistically significant (P >0.99).

| Family | Taxonomy | Combined Abundance | PASTURE Abundance | STALL Abundance |
|---|---|---|---|---|
| *Melthanobacteriaceae* | Archaea: *Euryarchaeota; Methanobacteria; Methanobacteriales, Methanobacteriacea* | 50,458 | 44,310 | 6,148 |
| *Methanocorpusculaceae* | Archaea: *Halobacterota: Methanomicrobia;, Methanomicrobiales; Methanocorpusculacea* | 1,330 | 987 | 343 |
| *Methanomethylophilaceae* | Archaea: *Thermoplasmatola;, Thermoplasmata; Methanomasilicoccales; Methanomethylophilacea* | 132 | 113 | 19 |

**Table** I. Absolute abundance (number of reads) of methanogen families in relation to animal housing type.



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The relative abundance of OTUs at the level of the methanogen families (i.e., *Methanobacteriaceae*,

An overview of the whole microbiota composition of pasture and stall cattle is presented in Figure 1.

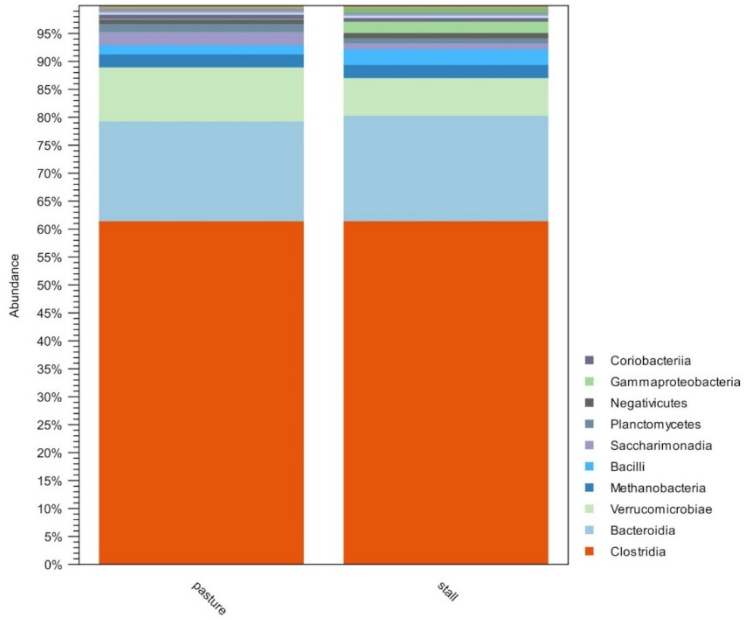


**Figure** 1. Bar Plots showing class-grouped OTU abundance (%) of cattle microbiota according to housing type. The legend indicates the ten most abundant entries.

The relative abundance of OTUs at the level of the methanogen families (i.e., *Methanobacteriaceae*, *Methanocorpuscolaceae*, *Methanosarcinaceae*, *Methanomethylophilaceae*) aggregated by housing type, is shown in Figure 2, where data from all sampling sites (Northern, Southern, Eastern, Western, Central regions of Kazakhstan) are grouped. The bioinformatics analysis revealed that the relative abundance of representatives from the *Methanobacteriaceae, Methanocorpuscolaceae, Methanosarcinaceae*, and *Methanomethylophilaceae* families was higher in animals housed on pasture compared with stall.

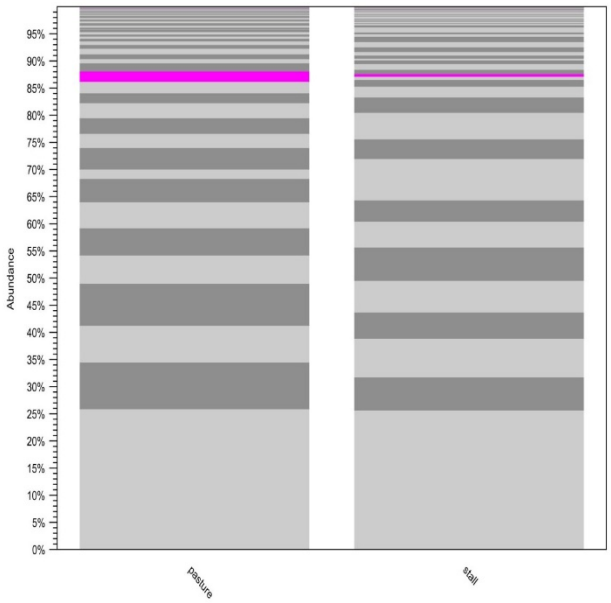


**Figure** 2. Bar plots showing family-grouped OTU abundance (%) of cattle microbiota according to housing type. Methanogens (Methanobacteriaceae, Methanocorpuscolaceae, Methanosarcinaceae, Methanomethylophilaceae) are highlighted in violet.



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Alpha diversity was significantly higher in animals during the pasture period compared to animals in the stall period (p-value = 0.002) (Figure 2).

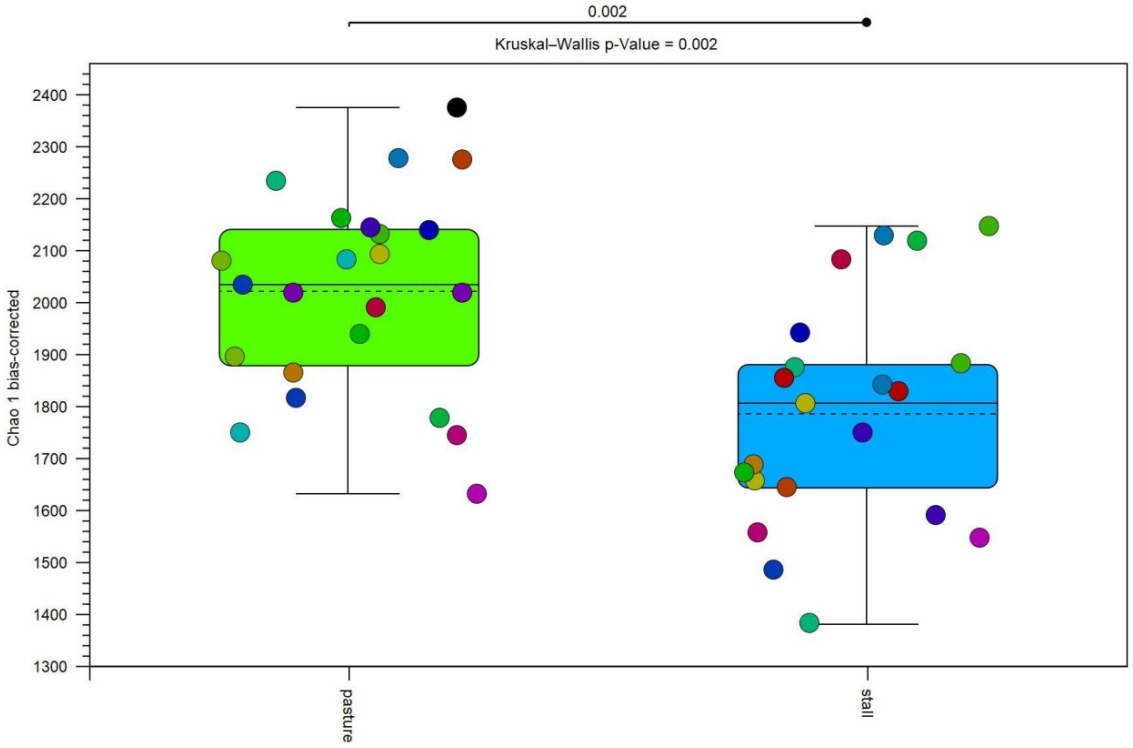


**Figure** 3. Alpha diversity of cattle microbiota calculated with bias-corrected Chao-1 index according to housing type.

Beta-diversity analysis in methanogens at the OTUs level between pasture and stall housing revealed a tendency to cluster, even though discrete separation of the two housing groups was not observed (Figure 3).

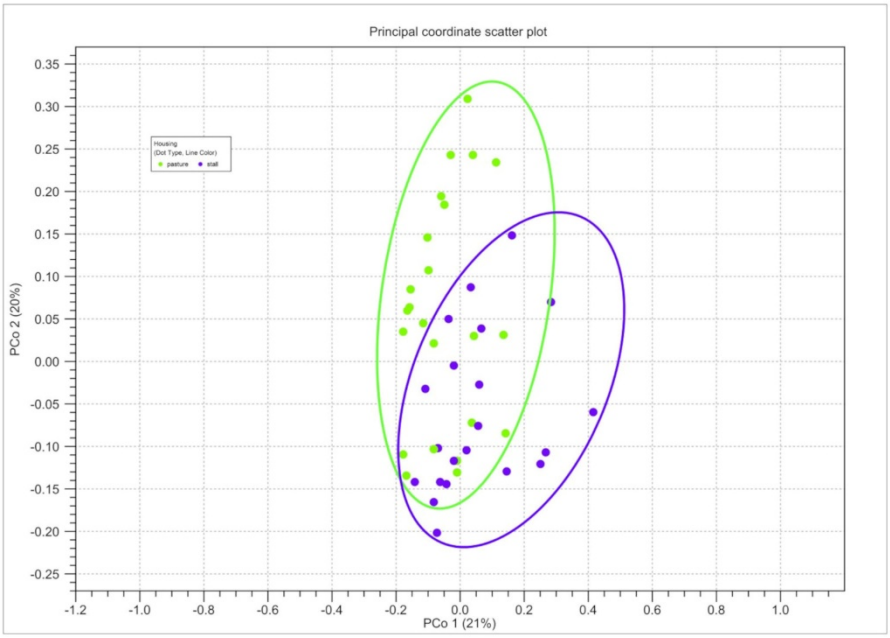


**Figure** 4. Scatter plot of the Beta diversity comparing housing type (green - pasture; violet - stall).



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Scatter plots depicting PCoA beta-diversity of pasture and stall samples are presented in Figure 3. PERMANOVA analysis accounting for the variable housing (pasture/stall) confirmed significant differences at the family level for archaeal methanogens (Table II).

| Variable | Groups | Pseudo-f statistic | p-value | p-value (Bonferroni) |
|---|---|---|---|---|
| | | **Bray-Curtis** | | |
| Housing | pasture. stall | 4.52693 | 0.00257 | 0.00257 |
| | | **Jaccard** | | |
| Housing | pasture. stall | 3.24410 | 0.00282 | 0.00282 |
| | | **Euclidean** | | |
| Housing | pasture. stall | 8.13633 | 0.00108 | 0.00108 |

**Table** II. PERMANOVA analysis of beta diversity.

| Family | PASTURE vs. STALL | | | | | |
|---|---|---|---|---|---|---|
| | Max group mean | Log2 fold change | Fold change | p-value | FDR p-value | Bonferroni |
| *Methanobacteriaceae* | 1,926.52 | 2.15 | 4.45 | 1.34E-4 | 1.22E-3 | 0.02 |
| *Methanomethylophilaceae* | 4.91 | 1.55 | 2.93 | 0.03 | 0.12 | 1.00 |
| *Methanocorpusculaceae* | 42.91 | 0.44 | 1.36 | 0.50 | 0.75 | 1.00 |
| *Methanosarcinaceae* | 0.04 | 0.28 | 1.26 | 0.83 | 0.90 | 1.00 |

**Table** III. Differential analysis of the abundance of methanogen families.

From these data, it is evident that even the most conservative p-value (Bonferroni-corrected) still remains significant (p-value = 0.02), indicating a considerably higher abundance of the *Methanobacteriacea* family in animals in the pasture period compared to those in the stall period. The relative abundance of *Methanabacteriaceae* family is 4.45-fold higher in pasture animals compared to one housed in stall. Additionally, unadjusted p-values are also significant for *Methanomethylophilaceae* family (p-value = 0.03).

Table IV shows all families that were found to be differentially abundant in pasture and stall animals. Most families showing significant differences were more abundant in the stall period, with the exclusion of *Erysipelatoclostridiaceae, Planococcaceae,Morganellaceae, Saccharimonadaceae*, and, as already reported, *Methanobacteriaceae*.



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| Family | PASTURE vs. STALL | | | | | |
|---|---|---|---|---|---|---|
| | Max group mean | Log2 fold change | Fold change | p-value | FDR p-value | Bonferroni |
| *Clostridiaceae* | 595.43 | -4.73 | -26.49 | 2.47E-24 | 4.25E-22 | 4.25E-22 |
| *Erysipelatoclostridiaceae* | 166.83 | 4.71 | 26.14 | 1.34E-12 | 1.01E-10 | 2.31E-10 |
| *Bifidobacteriaceae* | 33.67 | -5.63 | -49.62 | 1.77E-12 | 1.01E-10 | 3.04E-10 |
| *Pseudonocardiaceae* | 32.29 | -5.68 | -51.33 | 4.12E-9 | 1.77E-7 | 7.08E-7 |
| *Thermoactinomycelaceae* | 11.62 | -6.42 | -85.84 | 1.28E-8 | 4.42E-7 | 2.21E-6 |
| *Streptococcaceae* | 281.29 | -4.26 | -19.19 | 1.60E-8 | 4.60E-7 | 2.76E-6 |
| *Peptostreptococcaceae* | 4,794.52 | -2.80 | -6.96 | 3.28E-8 | 8.06E-7 | 5.65E-6 |
| *Corynebacteriaceae* | 11.43 | -4.40 | -21.18 | 7.61E-8 | 1.64E-6 | 1.31E-5 |
| *Planococcaceae* | 111.35 | 4.02 | 16.20 | 1.79E-7 | 3.42E-6 | 3.08E-5 |
| *Porphyromonadaceae* | 3.52 | -4.81 | -28.11 | 9.45E-7 | 1.62E-5 | 1.62E-4 |
| *Alcaligenaceae* | 2.00 | -5.02 | -32.34 | 7.36E-6 | 1.06E-4 | 1.27E-3 |
| *Micrococcaceae* | 22.00 | -3.70 | -13.00 | 1.09E-5 | 1.42E-4 | 1.87E-3 |
| *Morganellaceae* | 13.43 | 6.09 | 67.89 | 1.16E-5 | 1.42E-4 | 1.99E-3 |
| *Ruminococcaceae* | 3,135.81 | -1.30 | -2.47 | 3.65E-5 | 4.12E-4 | 6.28E-3 |
| *Dietziaceae* | 2.90 | -3.86 | -14.51 | 3.83E-5 | 4.12E-4 | 6.59E-3 |
| *Lactobacillaceae* | 6.29 | -3.66 | -12.66 | 4.19E-5 | 4.24E-4 | 7.20E-3 |
| *Methanobacteriaceae* | 1,926.52 | 2.15 | 4.45 | 1.34E-4 | 1.22E-3 | 0.02 |
| *Saccharimonadaceae* | 2,114.13 | 1.66 | 3.16 | 2.38E-4 | 2.05E-3 | 0.04 |

**Table** IV. Families differentially abundant when comparing the microbiota of pasture and stall cattle.

Figure 4 shows a heat map reporting the differential abundance of families for individual accessions (red = more numerous; blue = less numerous). In addition, this figure also shows the phylogenetic relationships between families.

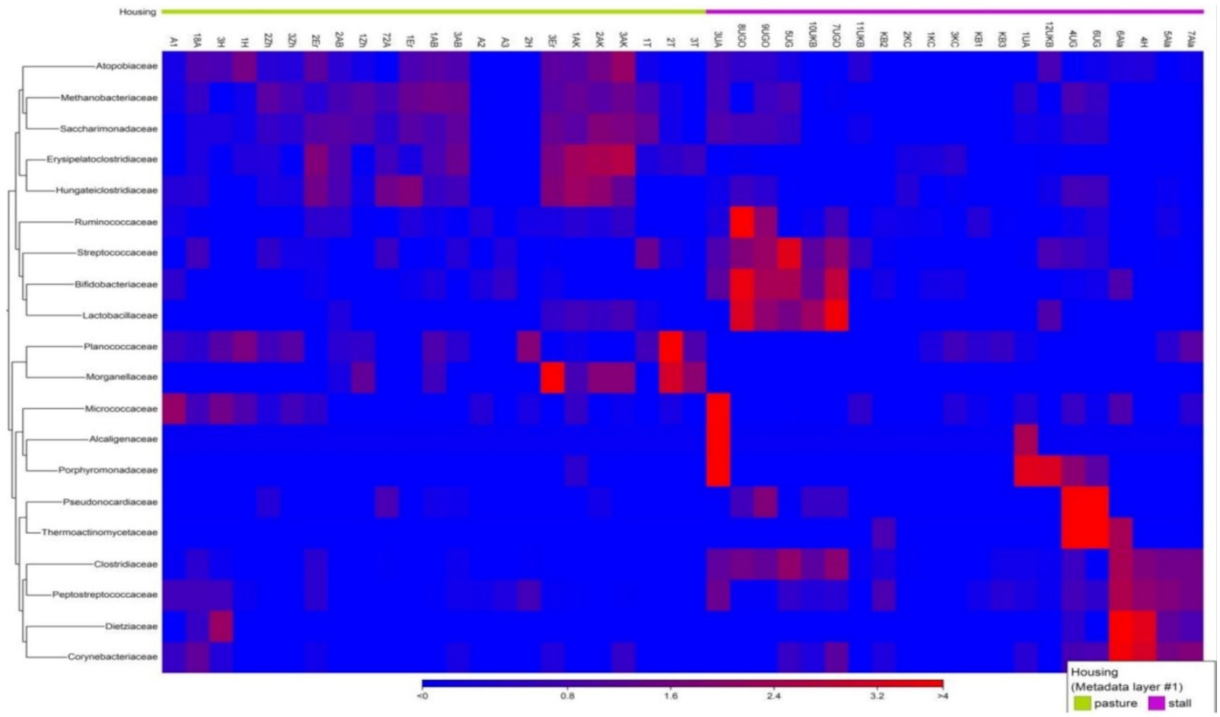


**Figure** 5. Heat-map of microbial community composition with cluster analysis. The analysis includes the families resulted to be differentially abundant in stall and pasture cattle. The color intensity in each panel shows the relative abundance in a sample, referring to color key at the bottom.



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## Discussion

As a result of the comparative studies carried out on cattle, it was found that the concentration of methanogens in the fecal contents of animals on pasture was higher compared to those in the stall period**.** Consequently, in diets with higher concentrations quantities, an increase in propionate occurs, leading to a subsequent decrease in methane emissions. (Wallace et al., 2019). When the proportion of concentrate in the diet of animals is increased, the rumen pH decreases, thereby inhibiting the growth of methane-producing bacteria and ciliates, while concurrently increasing the production of propionic acid (Demeyer and Henderickx 1967). This leads to a diminished availability of hydrogen required for methane formation, ultimately limiting methane emission. Moreover, with an increased concentrate in ruminant diets, there is not only an elevation in propionic acid, but also a reduction in acetic acid, positively impacting feed efficiency and animal performance. In fact, propionic acid is primarily converted into body composition by the liver supplying energy for crucial processes such as reproduction, growth, milk production and meat production. Due to the inverse functional relationship between methane emissions and propionic acid production, controlling concentrate-to-forage ratio, not only reduces methane emissions but also increases the overall productivity of ruminants. For example, diets with high herb contents, as fed during the dry period, usually lead to greater methane production in the rumen compared to those with high grain content (Bach et al., 2018).

A recent meta-analysis of *in vitro* tests showed a greater accumulation of $H_2$ when inhibiting melanogenesis in incubations with an increase in the concentration substrate. It has been hypothesized that animals receiving hay will accumulate a smaller amount of $H_2$ compared to those receiving hay – concentrate, due to the transition of fermentation to reducing processes, which consume more reducing equivalents, ultimately leading to less energy loss of the animal (Martinez-Fernandez et al., 2016). In addition, Vyas et al., also showed an increased release of $H_2$ in animals with suppressed methanogenesis which received a diet with a high content of concentrates, compared with a hay-concentrates mixture (Vyas et al., 2016). In two *in vitro* studies, a greater accumulation of $H_2$ was detected during the inhibition of methanogenesis in fermentations of mixed coarse feed with concentrates compared with a coarse substrate (Lin et al., 2013; O'Brien et al., 2013). Switching from mixed animal diets to livestock grazing can increase $CH_4$ production, as the forage-to-concentrate ratio generally increases $CH_4$ emissions (Aguerre et al., 2016). Methane emissions are highly dependent on dietary fiber source (Hammond et al.,, 2016). At the same time, other researchers did not find a difference in methane emission from dairy cattle depending on the periods of stall and pasture housing (Szalanski et al., 2016).

A higher number of *Clostridiaceae* were also found in stall animals, in contrast to pasture animals. *Clostridiaceae*are anaerobic spore-forming bacteria. Most *Clostridiaceae* are commensals and are important for the digestion of carbohydrates and proteins, and many species are involved in the metabolism of bile acid. Some *Clostridiaceae*, such as *Clostridium perfringens*, are associated with diseases. Our study confirms the data of Liu et al.,, who indicated that the abundance of *Bifidobacteriaceae* was significantly higher in grain-fed animals. However, it is contradicted that *Porphyromonadaceae* were the main distinguishing features in the grass-eating group (Liu et al., 2020). Our results are consistent with those of Wang et al., who found that higher levels of human fecal methanogenic microbiota were significantly associated with higher alpha diversity and showed differences in composition compared to low levels of methanogenic microbiota (Wang et al., 2022).

The intestinal content composition is largely influenced by the type of feeding, the composition of the diet and the included feed. Grazing animals contribute to an increase in the amount of methanogens, thereby leading to higher methane emissions. To reduce methane emissions, we recommend feeding grazing animals with concentrates in the evening.

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