Diversity of Gut Methanogens and Functional Enzymes Associated With Methane Metabolism in Smallholder Dairy Cattle

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Abstract

Methane is a greenhouse gas with disastrous consequences when released to intolerable levels. Ruminants produce methane during gut fermentation releasing it through belching and/or flatulence. To better understand the diversity of methanogens and functional enzymes associated with methane metabolism in dairy cows, 48 samples; six rumen and 42 dung contents were collected and analyzed using a shotgun metagenomic approach. The results indicated archaea from 5 phyla, 9 classes, 16 orders, 25 families, 59 genera, and 87 species. Gut sites significantly contributed to the presence and distribution of various methanogens ($P<0.01$). The class Methanomicrobia was abundant in the rumen samples (~ 39%) and in dung (~44%). The most abundant (~17%) methanogen species identified was *Methanocorpusculum labreanum*. However, some taxonomic classes were not classified (~ 6% in the rumen and ~4% in the dung). Furthermore, five functional enzymes: Glycine/Serine hydroxy methyltransferase, Formylmethanofuran—tetrahydromethanopterin N-formyltransferase, Formate dehydrogenase, Anaerobic carbon monoxide dehydrogenase and Catalase-peroxidase were associated with methane metabolism. KO0600 module and Enzyme Commissions (1.11.1.6 & 2.1.2.1) were common for dung and rumen fluid's enzymatic pathways. Functional analysis for the enzymes identified were significant ($P<0.05$) for 5 metabolism processes. Breeding for tolerable methane emitting dairy cattle for a sustainable environment should be undertaken.

1 Introduction

The livestock industry contributes to the subsistence of more than one billion of the world's poorest people and supports approximately 1.1 billion people (Hurst et al., 2005). Rearing livestock is an effective risk mitigation strategy for vulnerable populations, and an important nutrient and traction source for smallholder farming systems (Thornton, 2010). Despite there being great disparities between developed and developing countries, livestock products make up 33% of consumed protein and 17% of consumed kilocalories globally (Rosegrant et al., 2009). It is estimated that, by 2050, milk and beef production will increase by 191 and 153 percent, respectively (FAO, 2018). This will lead to an increased need to improve productivity to cater for the increased food demand. However, producing food intensively is likely to place more burden on the environment, as it may increase the amount of greenhouse gases (GHG) produced by the animals.

Methane (CH_4) is a greenhouse gas with a 28-times global warming potential than that of carbon dioxide (CO_2) (IPCC et al., 2014). The main sources of methane emissions from anthropogenic activities are carbon dioxide and ruminants. The livestock sector contributes 14.5% of global GHG emissions (Gerber et al., 2013). In ruminants, CH_4 is produced after reduction of CO_2 using H_2 by methanogenic archaea (Danielsson et al., 2012). Methanogenic archaea are part of symbiotic microorganisms that aid in the breakdown of consumed complex carbohydrates from plants to simple molecules that can be utilized by the ruminant animals (Danielsson et al., 2012; St- Pierre & Wright, 2013). The microorganisms in this mutual association are bacteria, protozoa, and fungi. The host facilitates the microorganisms' physical and chemical requirements (Stewart et al., 2018), while the microbes break down complex compounds to

produce volatile fatty acids (VFA) and varying amounts of formic acid, hydrogen (H_2) and CO_2 (Hook et al., 2010). Methane production by the methanogenic archaea process holds H_2 partial pressure down, leading to lesser fermentation of reduced end products like acetate (Moss et al., 2008). The produced methane is not utilized by the animal itself; rather it is eructed from the animal to the atmosphere. Methane is therefore not only harmful to the environment, it also represents a proportion of 2 to 12% gross energy loss to the animal (Johnson & Johnson, 1995).

In recent years, the knowledge on gut microbiology has risen due to new molecular techniques such as next-generation sequencing, but the methanogens from the guts of crossbred dairy animals in developing countries have not been well characterized and their connection to CH_4 levels remains largely unaddressed. Moreover, given the differences in the physiological status of various sections of the cattle gut, it is unclear which microbial genes are responsible for methane production in these various sections of the gut of cattle reared by small holder dairy cattle and who are exposed to sharp fluctuations in feed(s). To address these questions, we studied cows reared by small holder dairy farmers in the tropics that had different genotypes and were reared as different herds to identify gut methanogens from the rumen fluid and dung samples and functional enzymes associated with methane production and metabolism.

2 Materials And Methods

2.1 Description of the experimental sites

The research was undertaken in one experimental site in Kenya (University of Nairobi (UON) Faculty of Veterinary Medicine, Kanyariri farm, Kiambu County) and two sampling sites in Tanzania (Rungwe and Lushoto districts). The Tanzanian sampling sites and herd were under a Tanzanian led Project by International Livestock Research Institute (ILRI) dubbed "*Maziwa Zaidi platform*".

2.2 Sample collection from the experimental animals

Individual fecal samples were collected from 18 crossbred dairy cows from each district in Tanzania and from six animals reared at the UON Veterinary and teaching farm. The Tanzanian's herd was purposefully selected based on the animal's genotypes (Cheruiyot et al., 2018), and willingness of the dairy farmers to participate in the research project. The collection of fecal samples was done by palpating the rectum using clean sterilized lubricated hand sleeves. About 250g of fecal matter was hand grabbed and a proportion transferred into a sterile labelled 50 ml falcon tube as described by Kibegwa et al., (2020). About 250ml of rumen fluid was collected using a flexible stomach tube from each animal of the six experimental animals at UON Veterinary and teaching farm immediately after collection of fecal samples. The first 200 ml of the rumen fluid was then discarded to avoid salivary contamination and the remaining 50 ml transferred into sterile labelled falcon tubes. The samples from each sampling were immediately placed in a cool box with ice cubes and shipped to the Biosciences East and Central Africa (BecA-ILRI) Hub laboratories, Nairobi campus, where they were stored at -20°C until further processing.

2.3 DNA Extraction and Illumina sequencing library preparation

Before DNA extraction, samples were thawed at room temperature and then whirl wound thoroughly at maximum speed for at least 30 seconds as described by Habimana et al., 2018 for homogeneity. Whole genomic DNA was extracted from individual samples using the QIAamp DNA Stool Mini Kit (Qiagen, USA) following the manufacturer's instructions. Nucleic acid quality and quantity was assessed using NanoDrop® ND-2000 UV spectrophotometer (Nano-Drop Technologies, Wilmington, DE) and 1% Agarose gel electrophoresis. Library construction was performed using the Nextera DNA Preparation Kit and Nextera index Kit (Illumina, San Diego, CA, USA), following the manufacturer's instructions. In summary, the procedure involved two PCR procedures. In PCR one, 50ng of the genomic DNA was initially fragmented and adapters added simultaneously. The second PCR was limited (5 cycles) PCR to amplify the fragments of interest. After the first PCR, the fragments were purified using the Zymo DNA Kit (Zymo Research Corporation, Irvine, CA, USA) while after the second PCR, the product was cleaned up and size selected using AMPure XP beads (A63881, Beckam Coulter, Brea, CA, USA). The final library concentration and the average library size was measured using the Qubit® HS Assay Kit (Life Technologies Corporation, Grand Island, NY, USA), and Bioanalyzer tapestation 2200 (Agilent Technologies, Santa Clara, USA), respectively. Paired end (200 cycles) sequencing of pooled equimolar libraries was done using the Illumina MiSeq v3 (Illumina) System at the Biosciences eastern and central Africa (BecA)-ILRI Hub.

2.4 Methanogens taxonomic characterization and identification of enzymes and pathways associated with methane metabolism

All reads were uploaded to the ILRI research computing cluster (https://hpc.ilri.cgiar.org/), were analyzed and taxonomic assignment was done. Prior to taxonomic assignment, evaluation of sequence quality was done using FastQC software v.0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), poor quality reads were truncated using FASTX-trimmer, within the FASTX-toolkit v.0.0.14 (https://hannonlab.cshl.edu/ fastx.toolkit/). Sequences that had passed quality control were uploaded to MG-RAST (metagenomics Rapid Annotation using Subsystem Technology) for detection of taxa. Quality filtered sequences were then assembled into contigs using SPAdes version 3.13.0. (https://github.com/ablab/spades). SPAdes-assembled contigs were uploaded to Kyoto Encyclopedia of Genes and Genomes (KEGG) reference database (Kenahisa and Goto, 2000). Functional genes were identified from the database with the KEGG Automatic Annotation Server (KAAS) version 2.1 (KAAS query list) as previously shown by Auffret et al. (2017). The contigs from each sample were separately blasted to a dataset of prokaryotes based on the assignment method of a bidirectional best hit and then computed and the structural genes checked via https://www.kegg.jp. The KEGG IDs were picked and imported to the Ipath3 to visualize their involvement in metabolism of

microbial metabolism in diverse environment(s). The statistical analysis was performed using IBM SPSS statistics 20 at P<0.05.

3 Results

Generally, the relative total abundance (%) of the methanogens from dung samples were Kenyan dung (43.56%), Lushoto (30.78%), and Rungwe (9.76%). Taxonomic classes Archaeoglobi, Thermococci, and Thermoprotei showed relative abundance only in the dung and not in the rumen for the case of Kenyan samples (Fig. 1).

The taxonomic class Methanomicrobia was dominant (~39%) from the rumen and from the dung samples (~44%) (**Supplementary File 1** and **2**). Taxanomic classes: Methanomicrobia and Methanobacteria had more abundance of the species in the dung when compared to their presence in the rumen. The other taxonomic classes had a higher abundance in the rumen other than in the dung (**Supplementary File 3**). The 5 phyla identified according to the relative abundance during the study were Euryarchaeota (95.37%), Crenarchaeota (3.74%), Thaumarchaeota (0.52%), Korarchaeota (0.31%) and Nanoarchaeota (0.06%) (**Supplementary File 4**).

This study identified some species that were candidates for taxonomic classification. A higher relative abundance (%) of the candidates were from Tanzanian samples (Fig. 2). Some species were noted missing out to the higher taxonomic class but got assigned at the genus level. A proportion of ~6% of the rumen samples were unclassified, while ~4% of the dung samples were not classified. At the lower level of classification, averagely ~7.77% (at the genus level) of the species remained unassigned. Of the unassigned Archaeal methanogens (at the genus level), Kenyan samples had a collective relative abundance of 7.04% (dung) and 9.94% (rumen) while Tanzanian had a proportion of 7.7% (Lushoto) and Rungwe 7.75% (Fig. 3). Most of the unassigned archaeal species were from the genera Cenarchaeum and Nitrosopumilus. The Class Aciduliprofundum was noted to have candidates for classification. The scholars Duya et al., (2020) and Wemheuer et al., (2019) during their studies identified the taxonomic family Nitrosopumilaceae being dominant, which was different during this study as members of the said family had species who are not fully classified.

Methanogens from 5 phyla (Archaeal domains) related to methane release and metabolism were identified. They were Euryarchaeota, Crenarchaeota, Thaumarcheota, Korarchaeota, and Nanoarchaeota. Crenarchaeota and Euryarchaeota phyla were statistically significant (P< 0.05) in the rumen and dung samples. Methanogens from the following 3 genera; *Methanocorpusculum, Methanosarcina*, and *Methanococcus* were statistically significant (Table 1).

Table 1
Analysis for the presence of methanogens from the Rumen Fluid versus dung samples from Kenya

Phylum/Genus	Rumen	Dung	P-value
Crenarchaeota	4.6±0.15	2.67±0.17	<0.01
Sulfolobus	0.58±0.1	1.05±0.14	0.03
Pyrobaculum	0.45±0.06	0.47±0.13	0.91
Thermofilum	0.35±0.02	0.53±0.1	0.13
Staphylothermus	0.14±0.03	0.28±0.11	0.26
Caldivirga	0.15±0.05	0.17±0.08	0.85
Euryarchaeota	94.6±0.3	96.61±0.2	<0.01
Methanobrevibacter	20.62±2.31	14.74±1.34	0.07
Methanocorpusculum	22.01±2.51	4±0.22	<0.01
Methanosarcina	10.25±0.49	14.05±0.65	<0.01
Methanococcus	6.16±0.11	8.49±0.71	0.02
Methanothermobacter	3.39±0.14	3.07±0.17	0.19
Thaumarchaeota	0.45±0.2	0.52±0.07	0.75
Cenarchaeum	0.14±0.05	0.1±0.04	0.53
Nitrosopumilus	0.38±0.05	0.35±0.19	0.88
Korarchaeota	0.31±0.13	0.15±0.02	0.3
Nanoarchaeota	0.04±0.02	0.04±0.02	0.97

No variation ($P<0.05$) in the relative abundance of methanogens in the dung from the study areas was noted (Table 2 and Table 3).

Table 2
Analysis of dung methanogens in Kenya versus Tanzania analysis

Phylum/Genus	Kenya	Tanzania	P-value
Crenarchaeota	2.59±0.22	4.45±0.39	0.18
Sulfolobus	1.05±0.14	1.44±0.18	0.49
Pyrobaculum	0.47±0.13	0.73±0.09	0.34
Thermofilum	0.53±0.1	0.33±0.05	0.17
Staphylothermus	0.28±0.11	0.28±0.06	0.99
Caldivirga	0.17±0.08	0.26±0.05	0.54
Euryarchaeota	96.63±0.28	94.44±0.44	0.17
Methanobrevibacter	14.74±1.34	25.38±2.7	0.2
Methanocorpusculum	4±0.22	6.39±0.98	0.42
Methanosarcina	14.05±0.65	12.83±0.82	0.63
Methanococcus	8.49±0.71	6.32±0.39	0.08
Methanothermobacter	3.07±0.17	3.85±0.29	0.38
Thaumarchaeota	0.57±0.06	0.54±0.06	0.88
Cenarchaeum	0.1±0.04	0.26±0.05	0.31
Nitrosopumilus	0.35±0.19	0.28±0.05	0.66
Korarchaeota	0.16±0.03	0.49±0.08	0.23
Nanoarchaeota	0.04±0.02	0.08±0.03	0.72

Table 3
Analysis of the dung methanogens in the Tanzanian samples.

Phylum/Genus	Rungwe	Lushoto	P-value
Crenarchaeota	4.68±0.63	4.21±0.48	0.56
Sulfolobus	1.4±0.26	1.48±0.27	0.84
Pyrobaculum	0.74±0.1	0.72±0.14	0.93
Thermofilum	0.29±0.07	0.37±0.06	0.38
Staphylothermus	0.22±0.06	0.34±0.1	0.32
Caldivirga	0.3±0.07	0.21±0.05	0.36
Euryarchaeota	94.19±0.66	94.69±0.61	0.58
Methanobrevibacter	22.4±3.43	28.36±4.16	0.28
Methanocorpusculum	7.83±1.73	4.95±0.81	0.14
Methanosarcina	13.2±1.21	12.45±1.12	0.66
Methanococcus	6.75±0.57	5.88±0.51	0.27
Methanothermobacter	3.48±0.41	4.22±0.39	0.2
Thaumarchaeota	0.52±0.08	0.56±0.1	0.76
Cenarchaeum	0.28±0.08	0.24±0.06	0.73
Nitrosopumilus	0.28±0.07	0.28±0.06	0.96
Korarchaeota	0.54±0.12	0.43±0.1	0.47
Nanoarchaeota	0.06±0.03	0.1±0.06	0.54
Significant ($P<0.05$) variation among methanogens (16 species) in the rumen and dung was also noted (Table 4).			

Table 4
Significantly varying methanogen species between rumen and dung samples

Phylum/Species	Rumen	Dung	P value
Euryarchaeota			
Methanobrevibacter smithii	8.08±0.92	12.35±1.01	0.02
Methanosarcina acetivorans	5.82±0.38	4±0.35	0.01
Methanocorpusculum labreanum	4±0.22	22.01±2.51	<0.01
Methanosarcina mazei	3.17±0.31	2.31±0.05	0.03
Methanospirillum hungatei	2.93±0.14	1.91±0.2	0.01
Methanococcoides burtonii	2.93±0.07	2.04±0.15	<0.01
Aciduliprofundum boonei	2.6±0.18	0.99±0.04	<0.01
Methanococcus vannielii	2.55±0.13	1.78±0.09	<0.01
Archaeoglobus fulgidus	1.98±0.42	0.86±0.06	0.04
Thermococcus kodakarensis	1.75±0.15	0.75±0.1	<0.01
Thermoplasma acidophilum	0.93±0.19	0.34±0.05	0.02
Thermoplasma volcanium	0.83±0.11	0.43±0.03	0.01
Haloterrigena turkmenica	0.8±0.13	0.35±0.08	0.03
Methanothermococcus okinawensis	0.3±0.04	0.12±0.03	0.01
Crenarchaeota			
Hyperthermus butylicus	0.33±0.05	0.14±0.02	0.01
Ignisphaera aggregans	0.31±0.03	0.13±0.01	<0.01

Generally, Fig. 4 shows the metabolic functions that were affected in the rumen and dung samples by archaea derived from Ipath3. The pathway contributed by the functionality of the rumen samples was denoted by a green color, that from the dung was red, and those that overlapped was shown by the blue color.

Green represents functionality in the rumen affected by the enzymes generally, Blue represents the overlap of both the rumen and the dung and red represents dung functional enzymes in the metabolic pathways

Fig. 5 shows the enzymes of importance that functioned and/or affected in the samples from the dung within the methane metabolism pathways. The 5 enzyme commissions (EC) were: ECs 1.11.1.6, 2.1.2.1, 1.2.99.2 (Now referred as 1.2.7.4), 2.3.1.101 (Now referred as 1.17.1.9) and 1.2.1.2. The specific enzyme commissions were denoted by red stars on the box ECs. Enzymes with functionality in the rumen during

methane metabolism were shown in **Fig. 6** and the specific functional ECs (1.11.1.6 and 2.1.2.1) are shown by the red stars. The two functional ECs in the rumen and the KO0600 (methane metabolism) module pathway were common for both the rumen and dung samples.

The module KAAS database also showed functionality for metabolism (09100) and energy metabolism (09102). Modules describing methanogenesis using methanol (MOO356), acetate (MOO357), and methylamine/ dimethyamine (MOO563) or Carbon (IV) Oxide (MOO567) as in the KEGG pathway were noted alongside the stated ECs above. KEGG Orthology (KO) in the rumen were 271 ID entries while the KO in the dung were 411 IDs. The entry IDs had different metabolic functions. On the functional analysis level for 2 selection criteria of MGRAST, Fig. 7 shows 5 statistically significant ($P<0.05$) metabolisms both for the rumen fluid and dung samples that were generated. They are carbohydrate metabolism, biosynthesis of other secondary metabolites, xenobiotics biodegradation and metabolism, nucleotide metabolism, and amino acid metabolism. Bolded values were statistically significant ($P<0.05$) for the functional level 2 from the MGRAST analysis

4 Discussion

4.1 Gut Methanogens

Methanogens are microorganisms initially exclusively thought to be from the phylum Euryarchaeota. From the recent documentation, there has been advancement to other different phyla. Some of the phyla already agreed upon are Thaumarchaeota, Aigarchaeota, Crenarchaeota, Korarchaeota (TACK), Nonarchaeota, Bathyarchaeota, Geoarchaeota, Marsarchaeota, and Verstraetearchaeota (Berghuis et al., 2019; Vanwonterghem et al., 2016; Evans et al., 2015; Kelly et al., 2011). This study identified species from TACK, Euryarchaeota, and Nonarchaeota. Phyla Euryarchaeota and Crenarchaeota were noted to be dominant among archaeal methanogens and a few presences of methanogens from the phyla Thaumarchaeota, Korarchaeota and Nanoarchaeota. The same methanogen abundance was noted by Jia et al., (2017). Jia et al., (2017) further noted that the phylum Euryarchaeota (specifically those from the genus *Methanosarcina*) was notably responsible for a variety of functionalities related to methane biosynthesis. The genus *Methanosarcina* possibly will produce methane with the help of enzymes and protein constituents in the methyl nutrient pathway, acetic acid, and CO_2 reduction (Thauer, 2011). Methanogens occupy various diverse environments ranging from hostile environments to favorable conditions. They are acidophilic mesophiles and/or psychrophiles (Evans et al., 2015). Their vast environmental exposure would cause them to utilize different types of metabolism for their nourishment. Some of the phyla were not identified during this study because of the high temperature requirement that the gut of the bovines do not offer as their optimal conditions are between $38.8^{\circ}C \pm 0.5^{\circ}C$ (Chen et al., 2018) and/or their full information and categorization has not been fully documented. The Phylum Bathyarchaeota, for example, which was initially identified as Miscellaneous Crenarchaeotal group, has a small proportion of its species cultured and their genomic information completed, calling for advance work (Dayu et al., 2020; Meng et al., 2014; Lloyd et al., 2013).

Metharchaeal methanogens have been exclusive studied in comparison to other phyla. They have been recorded to have 155-200 isolated species which are clustered into 4 classes, 7 orders, 14 families, and 29-35 diverse genera (Singh et al., 2011). This study showed presence for 3 taxonomic classes, 6 orders, 12 families, 24 genera and 37 individual species of the Metharchaeal methanogens. This is an illustration of a high representation of the metharchaeal methanogens in the study areas. Methanogens in the rumen and rectal area of the ruminant species vary in their population's organization, ecology, and their substrate sources (Knapp et al., 2014). Such variation could be because of the prevailing physicochemical properties (Dayu et al., 2020). The most dominant species in the fore and hindgut was noted to be *Methanocorpusculum labreanum (~17%)*, followed by other hydrogenotrophic methanogens which is in agreement with a study by Auffret et al., (2017) and Chen et al., (2014). The dominance of Methanocorpusculum can be explained by their ability to utilize a wide range of substrates such as acetate, H_2 + CO_2, formate, ethanol, 2-propanol, 2- butanol, or cyclo-pentanol. Methanocorpusculum relies on acetate as a growth feature and on peptone, tungstate, and nickel for their stimulatory (Rosenberg et al., 2014). Moreover, this genus can survive in a wide environment, temperature range of 15-60℃ and pH of 6.1-8.0 (Liu and Whitman, 2008), all of which can be achieved in the rumen.

Methanococcus are methanogens that were thought to be only isolated from the sea. However, that opinion since changed when they were found in other environments that are not like marine conditions (Tumbula and Whitman, 2003). These species are not associated with any disease on their hosts and are firmly anaerobic and hydrogenotrophic. They have distinct abilities to undertake sulfur metabolism (Liu et al., 2009). Most are mesophilic (require a temperature of between 20-45°C), and others are thermophilic (41-122°C) or hyperthermophilic (above 60°C) (Stetter, 2006). In this study, mesophilic species: *Methanococcus aeolicus, Methanococcus maripaludis, Methanococcus vannielii*, and *Methanococcus voltae* were identified. The species identified in this study were similar to those identified by Goyal et al., (2016).

Methanosarcina metarchaeals utilize the substrates acetate, H_2 +, CO_2, CO, methanol, methylamines, and metylmercaptoproprionate dismetylsulfide. They grow on a variety of substrates because they are notably cytochrome bound (Buan et al., 2011). The methanogens here operate in a temperature range of between 1°C - 70°C and a pH range of 4-10 and adapt to low hydrogen availability (Liu and Whitman, 2008). Methanosarcinas have a flexible metabolic pathway making their growth genes on one substrate easily deleted without affecting their subsequent growth on another available substrate(s). With this capability, methanosarcina's genetic analysis can be used to investigate how methanogens grow and participate in methane production along the known methane metabolism pathways (Kulkarni et al., 2009). *Methanosarcina acetivorans*, *Methanosarcina barkeri*, and *Methanosarcina mazei* were identified during this study.

The methanogen *Methanocorpusculum labreanum* species that hailed from Methanocorpusculum was identified during this study. This species was found both among the rumen fluid samples and dung samples from the Kenyan dairy cattle. This was in contrast with a study by Liu et al., (2012) in a study on Chinese sheep that identified sequences related to Methanocorpusculum species from sheep droppings

only but absent in the rumen of the same sheep. In another study by Luo et. al., (2013), the authors found out that *Methanocorpusculum* were dominant (60%) in the hindgut of captive *Ceratotheriumsimum*. Methanocorpusculum utilizes the substrate acetate, H_2 +CO_2, formate, ethanol, 2-propanol, 2- butanol, or cyclo-pentanol. Species from this genus were also identified from a wastewater bio digester (Oren, 2014). Identification of this species in the rumen indicated that they may have a wider niche than previously thought.

4.2 Functional enzymes associated with methane metabolism

Enzymes catalyze chemical reactions that are key for life functionality such as metabolism and digestion (Blanco and Blanco, 2017). Over the years, there is an advancement of knowledge on methane metabolism between methanogens and methanotrophic archaea with a universal display of the Methyl-coenzyme M reductase complex as a main enzyme in their pathways (Evans et al., 2019). The study identified the function of the enzyme Glycine/Serine hydroxy methyltransferase in the aspects of Amino acid transportation and metabolism/biosynthesis from module entry K00600. This was identified from the Enzyme commission (EC) EC: 2.1.2.1 and was associated with the structural gene *glyA*. Glycine/Serine hydroxy methyltransferaseis a vitamin reliant enzyme that catalyzes the reversible and conversion of L serine to glycine and tetrahydrofolate to 5, 10-Methylenetetrahydrofolate. Upon completion of the enzymatic reaction, it leads to the delivery of substantial carbon units to the cell (Edgar, 2005). Other studies have noted that this enzyme as well catalyzes glycine and acetaldehyde to form L-threonine with 4-trimethylammoniobutanal to form 3-hydroxy N^6, N^6, N^6-trimethyl-L-lysine (Schweitzer et al., 2009). Methanogens like *Methanococcus jannaschii*, which was identified in this study, have been shown to use the enzyme for amino acid biosynthesis (Tsoka et al., 2004).

Formylmethanofuran-tetrahydromethanopterin-N-formyl transferase enzyme was noted from the EC: 1.17.1.9 (Formerly EC. 2.3.1.101). The module entry involved was K00672. The enzyme was notably involved in energy production and conversion. Entry K00123 that is involved in the anaerobic selenocysteine-containing dehydrogenase was also noted and needed for energy production and conversion. The gene responsible for the enzyme is *Ftr*. Structural genes associated with the functionality of the enzyme were *fdoG*, *fdhF*, and *fdwA*. Formylmethanofuran-tetrahydromethanopterin N-formyltransferase enzyme catalyzes two notable substrates; methanofuran and 5-formyl-5-6-7-8-tetrahydromethanopterin (Wagner et al. 2016). Methanofuran is the key for methane formation from CO_2 by methanogens. CO_2 as a methanogenic substrate is initially reduced and activated to formyl-methanofuran (Wagner et al., 2016). Mesophilic methanogen (*Methanosarcina barkeri*) and thermophilic methanogens (*Methanopyrus kandleri*), that were also identified in this study, have shown functionality for the enzyme (Enzmann et al., 2018).

Anaerobic carbon monoxide dehydrogenase enzyme facilitates the metabolism of methanogens by the reversible interconversion between carbon monoxide and CO_2. The catalyzed reaction is vital for energy conservation and carbon fixation among methanogens, especially during the Wood-Ljungdahl pathway

(King and Weber, 2007; Borrel et al., 2016). Enzyme anaerobic carbon monoxide dehydrogenase of the catalytic subunit was noted during this study from the EC: 1.2.7.4 pathway, formerly EC. 1.2.99.2. The involved module entry was K00198. Hydroxylamine reductase (hybrid-cluster protein) together with the enzymatic function of anaerobic carbon monoxide dehydrogenase are vital for inorganic ion transport and metabolism energy production and conversion. The key genes involved were *cooS* and *acsA*.

Catalase-peroxidase enzyme is documented to be an inconsequential material in the antioxidant system in methanogens even for those aerotolerant, including species such as *Methanosarcina acetivorans* (Jennings et al., 2014). The catalase-peroxidase enzyme is a sturdy catalase with H_2O_2 as the contributor which releases O_2 (Vlasits et al., 2010) and molecules of water in a two-step reaction (Nandi et al., 2019). Methanogens in their environments are exposed to oxygen occasionally in aerobic situations, this would call in for the functionality of antioxidants to facilitate lowering levels of oxygen (Angel et al., 2012). Ma and Lu (2011) pointed out that some methanogens can withstand some levels of oxygen for some hours. The enzyme possessed by methanogens (although they are noted to be limited in the numbers- restricted to Methanosarcina species and Methanobrevibacter species) has been noted to have been acquired through a gene (*katG*) transferred laterally (Zamocky et al., 2012a). When catalase peroxidase enzyme has functionality in EC: 1.11.1.6, thereby it acts on hydrogen peroxide as an acceptor and not the functionality under EC. 1.11.1.21 of both catalase and peroxidase. Module entry K03782 was involved. Specifically, the Catalase peroxidase I is involved in catalyzing inorganic ion transport and metabolism. The gene involved is *katG*.

In conclusion, this study broadens our understanding on dominant methanogen species in Kenya and Tanzanian among smallholder cattle and functional enzymes associated with methane metabolism and production. The methanogen species abundances from these study areas in numbers/kind can be utilized exclusively or jointly as indirect selection criteria for methane mitigation. This calls for interdisciplinary cohesion and collaboration for fruitful achievements. Studies should be carried out to taxonomically categorize species missing out of place. Furthermore, every part of the gut (either fore or the hindgut) was capable of hosting methanogens. Targets for methanogens should entirely be towards the whole gastrointestinal tract. Furthermore, there is a need to target functional genes of the microbes and those of animals to achieve a friendly environment without affecting the animal's functionality. Animals who are less methane emitters should be bred to cut on their methane release from the gut. Further studies should be carried out to target pathways for tolerable methane emitter dairy cattle without hindering other necessary metabolic processes. The fraction of the methanogens that are yet to be fully classified should be carried out and a thorough study of their temperature, substrate(s), and pH should be noted.

Declarations

Author's contribution

FMK, RCB and CKG conceived and designed the experiment. FMK conducted the experiment. FMK and DKN analyzed the data. DKN wrote the manuscript. All authors read and approved the manuscript.

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Financial and non- financial Interests

All authors declare no financial and non-financial interests

Statement of Ethical statement and Animal Rights

This research study was performed under the University of Nairobi (UON), Faculty of Veterinary Medicine Institutional Animal Care and Use Committee (IACUC) accepted procedures. Experimental animals were handled professionally while exercising restraint to reduce on any discomfort and injury.

Conflict of Interest Statement

The authors of this piece of work declares no conflict of interest.

Data availability Statements

All the data that were generated and/ or analysed during the study are included in the article that has been published and those that are contained in the supplementary files.

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Figures

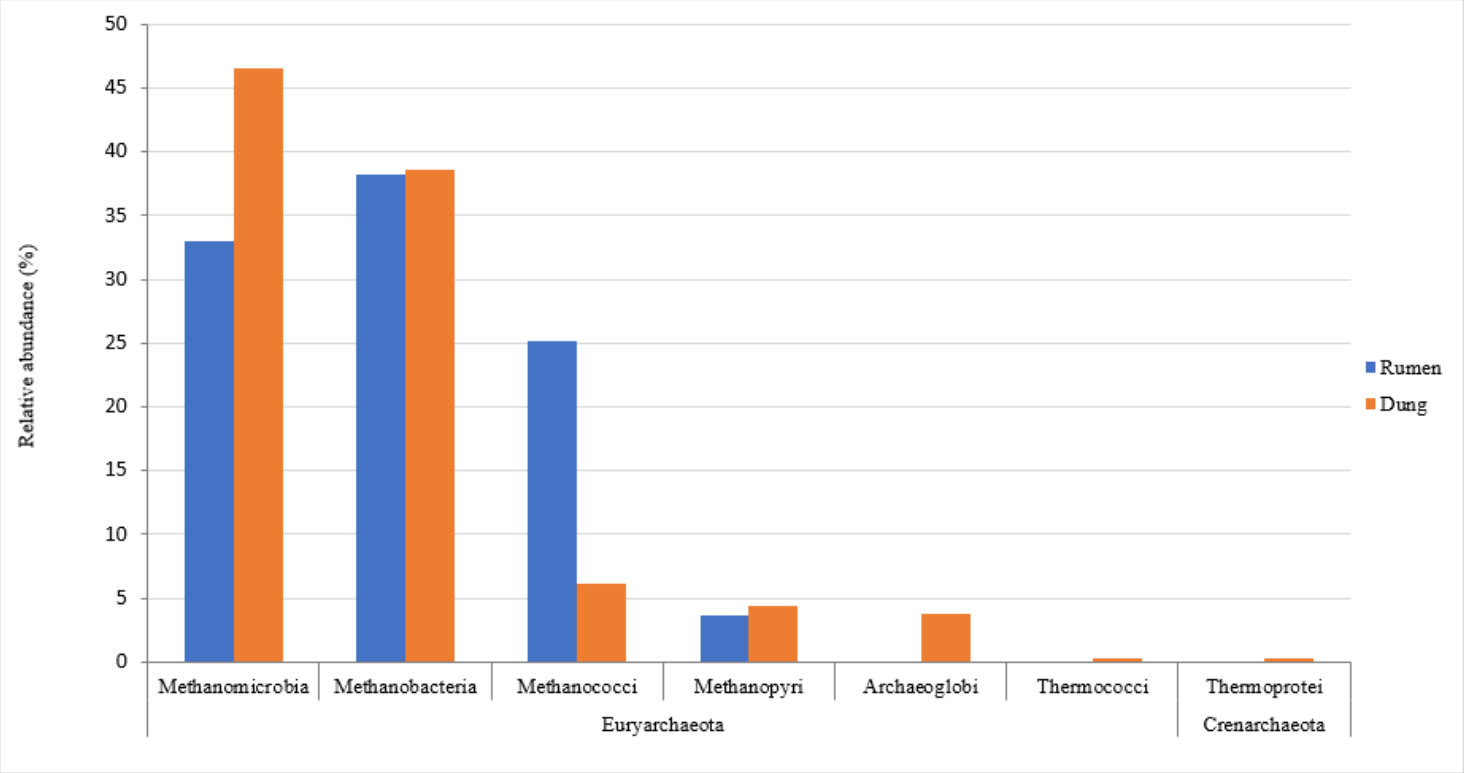

Figure 1

Relative abundance (%) of the Euryarchaeota and Crenarchaeota group from the rumen and the dung samples

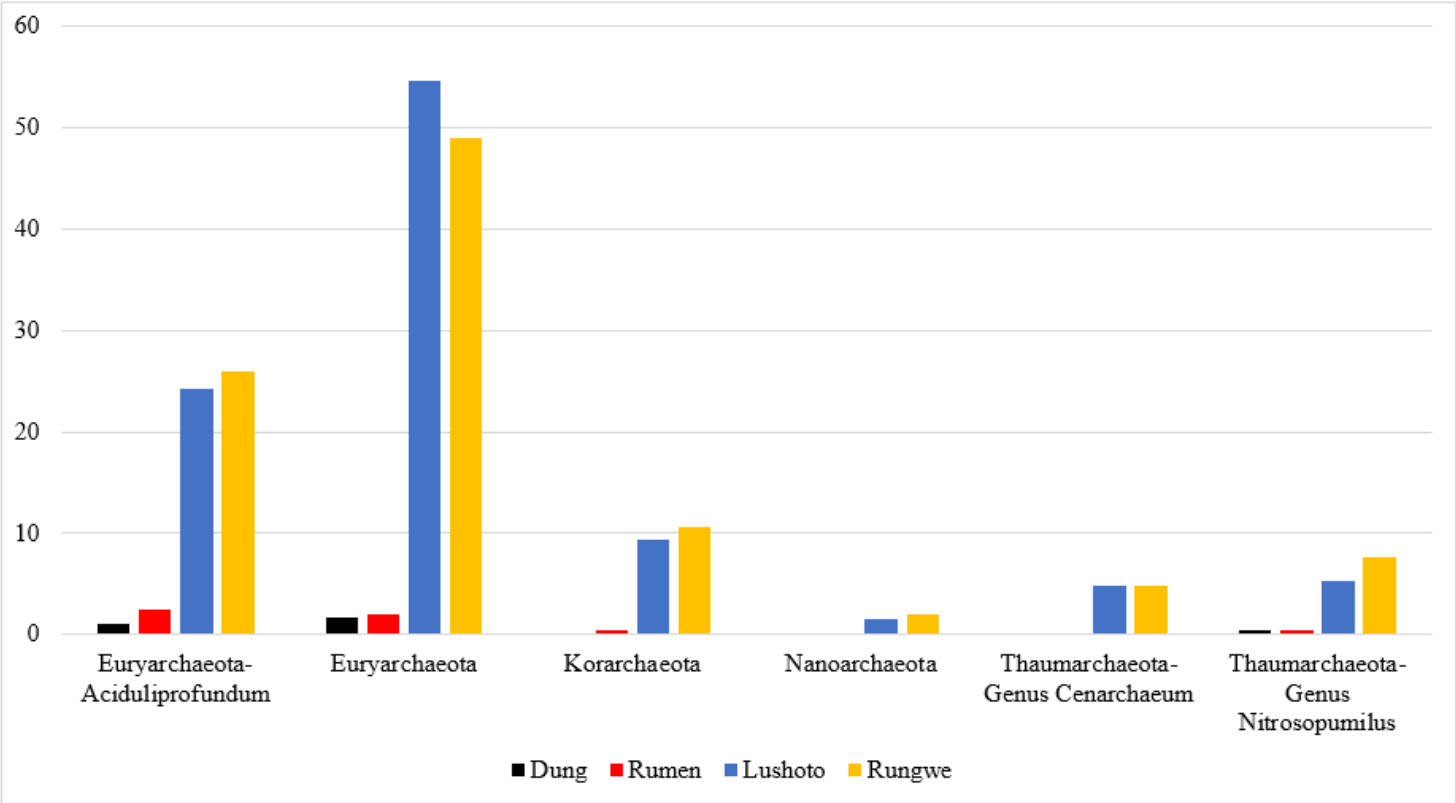

Figure 2

Relative abundance (%) of the unclassified methanogens from different phyla

Figure 3

Relative abundance (%) of the taxonomic classified and unclassified methanogens at the Genus level

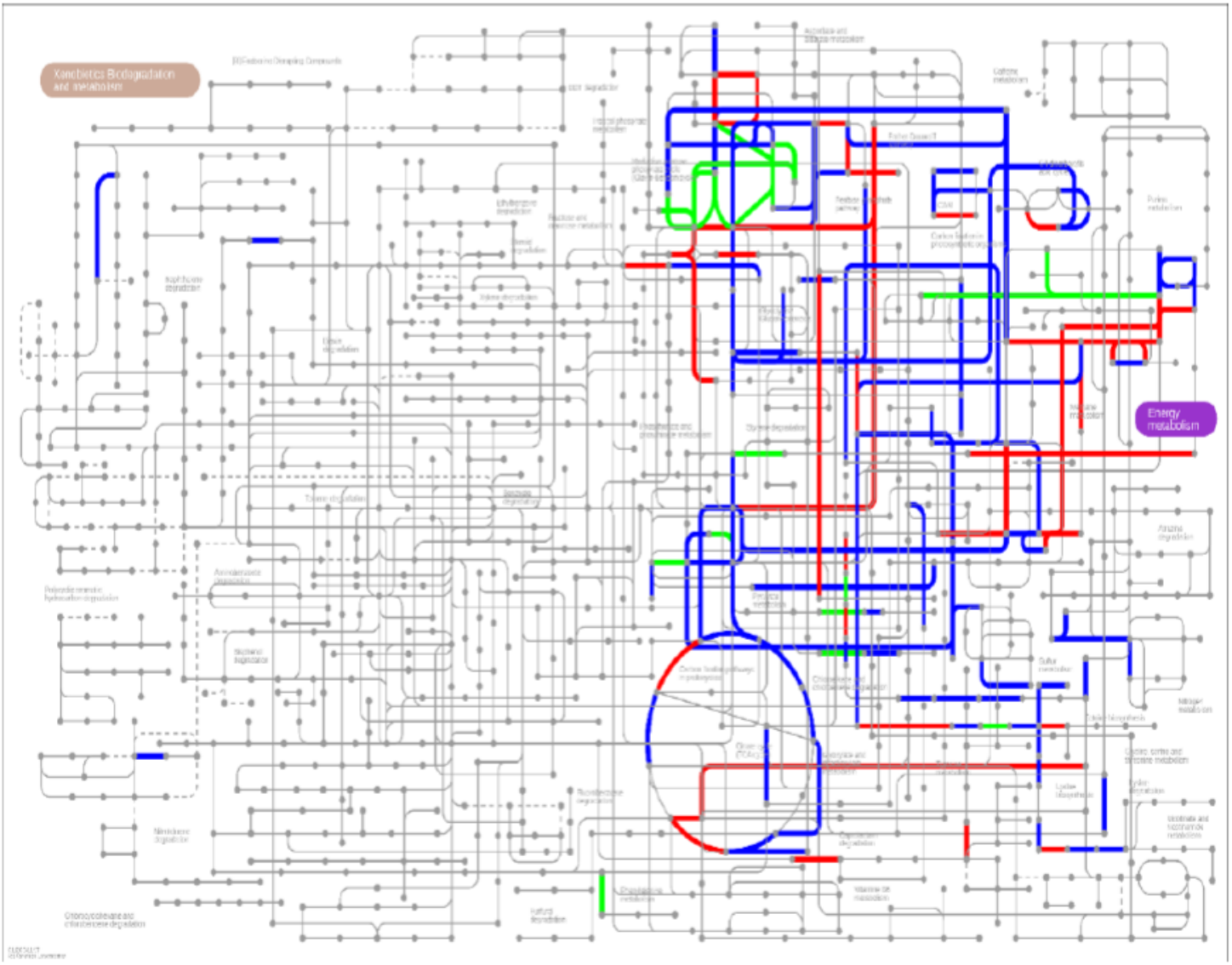

Figure 4

Ipath3 visualization of all metabolic functions affected by Archaea in rumen and fecal samples.

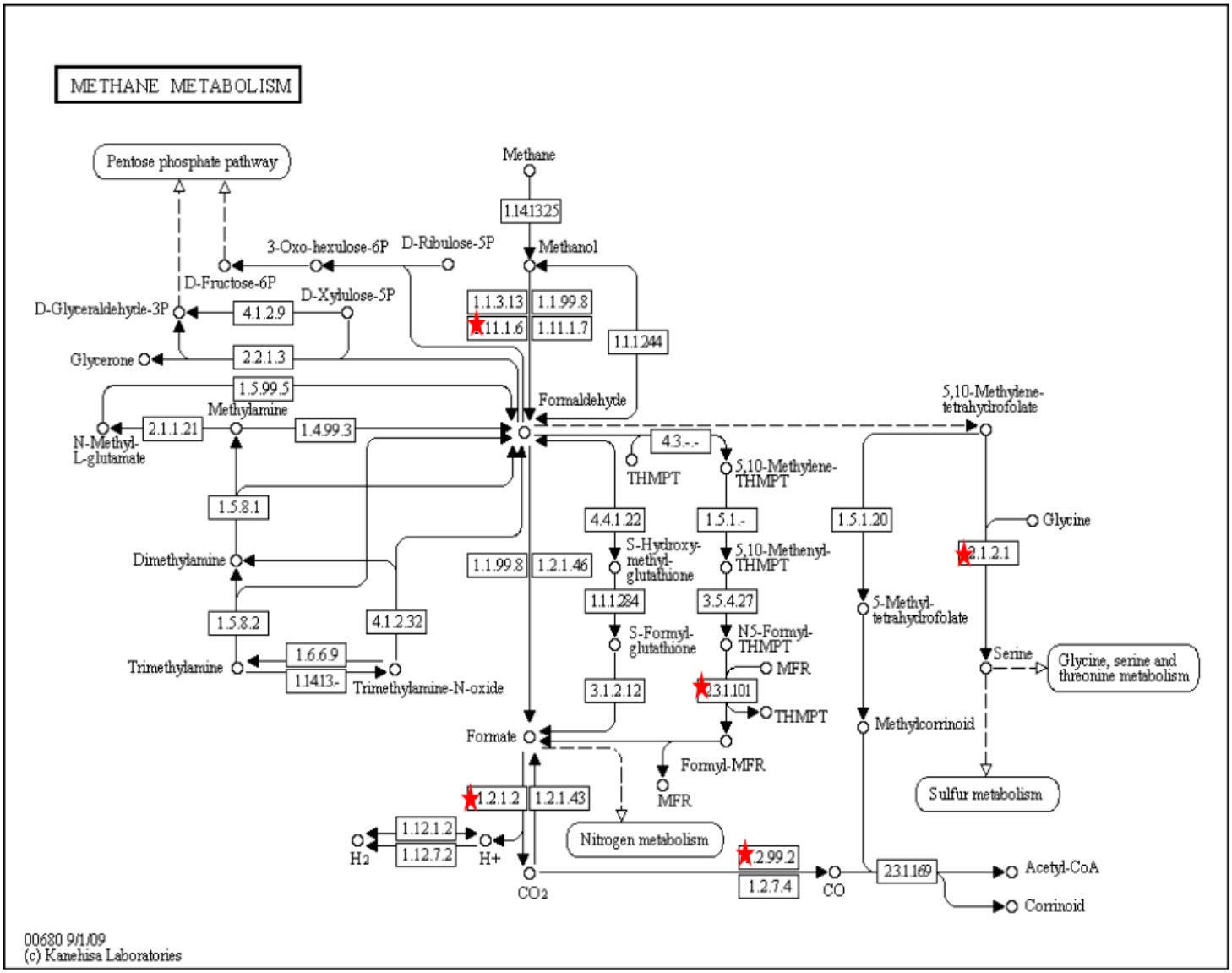

Figure 5

Enzymes affected in the dung samples within the methane metabolism pathway

Shows the Enzyme commissions affected in the pathway chart

Figure 6

Enzymes affected in the rumen fluid within the methane metabolism pathway

Shows the enzyme commissions affected during methane metabolism in the chart

Figure 7

Level 2 of KEGG Orthology (KO) functional analysis between rumen and fecal samples

Supplementary Files

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