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Metatranscriptome analysis to identify the influence of diet over metabolic proficiency of rumen in Mehsani buffalo

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Abstract

The rumen microbiome is a complex system of numerous microbes that play crucial role in digesting feed and are of particular interest for the extraction of enzymes for application in industries such as food, biofuel, and others. Metatranscriptome profiling gives insight into the genes being actively transcribed, providing a functional understanding of the rumen. Current study focuses over the analysis of rumen metatranscriptome samples from Mehsani buffalo, belonging to different diet treatments including dry and green roughage with varying amount of concentrate. The study aimed to determine the key biological processes in the rumen and how diet changes affect them. Results showed that the most prevalent microbial community was Prevotella and metabolic activities dominated, with carbohydrate metabolism, fermentation, and methanogenesis among the most abundant functional features. It was identified that functional profile of the rumen samples are majorly influenced by diet. The green roughage diet groups reflect high metabolic activity and contain genes for carbohydrate metabolism, which were not present in the dry roughage diet groups. Attempts were also taken to identify variation in fermentation and methanogenesis processes among these diet variations. Further, the study found that the type of diet greatly impacted the functional profile and have significant influence on metabolic capacity of animals. A set of 64 pathways were identified as significant ones in corresponds to diet variations and out of which 20 belongs to metabolic processes and this was further supported by differential abundance analysis, LEfSe and Random Forest analysis also, highlighting the importance of metabolism in rumen functionality. Metabolic pathways were more abundant in green roughage diets groups compared to dry roughage ones, indicating that green roughage stimulates more metabolic activity compared to dry roughage. Impact of concentrate in the diet was also visible. The study found that the type of diet, specifically the choice of roughage, significantly impacts the functionality of the rumen and affects its metabolic activity.

Keywords Rumen microbiome, Mehsani buffalo, Metatranscriptome, Roughage, Metabolic activity, Statistical analysis

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Introduction

Ruminants correspond to significant proportion of domesticated farm animal across the world and represents the major source of milk, meat, along with other dairy products [1]. The transformation of inedible materials for humans, including roughage, tree fodder, crop leftovers, and by-products, into edible food through the digestive process of ruminant animals remains a crucial aspect of animal agriculture. Researchers face both prospects and obstacles to enhance animal productivity by utilizing suitable technologies, particularly in the area of production systems, nutrition, and feeding [2]. Analyzing the impact of different ruminant feed is significant as it influences the ruminal microbial population structure and energy density, ultimately improving feed efficiency and average daily gain [3, 4]. Providing ruminants with fresh green roughage is a widespread practice worldwide, as it's a renewable source and meets the animals' protein, energy, mineral, and vitamin requirements. As an alternative, low-quality dry roughages, which are readily available, affordable, and nutritionally sufficient, are also fed to the animals to complete their maintenance needs [4, 5]. The possibilities of enhancing feed efficiency in ruminants by altering ruminal ecology requires further study and development, owing to its potential benefits [2].

Ruminants digest plant materials through an extensive microbial community [6–8] present in the rumen and provide nutrients to the host, mostly as volatile fatty acids and microbial protein [8, 9]. The rumen possesses a consortium of microbes that is responsible for the complex lignocellulosic degradation system for the attachment of microbes and plant biomass digestion. Though, the multifarious chemical processes required for the breakdown of the plant cell wall are generally not carried out by any single species [8].

The rumen microbiome is recognized as the most proficient and capable microbial system for degradation of lignocellulosic biomass [10], owing to which it has gained interest for mining enzymes for application in the feed along with food industry, cellulosic biofuel, and further industrial processes [11–14]. Rumen microbiome is extremely complex, and includes bacteria, fungi, protozoa, methanogens, and bacteriophages [12, 15]. These symbiont microbes have evolved with the host for millions of years in anaerobic conditions, high dilution rates and cell densities along with predation of protozoa [13]. This selective pressure has speed up the evolution of a microbiota as a community which is highly dedicated to lignocellulosic biomass degradation. The host is dependent over various enzymes, generated by the microbiome, for the conversion of composite fibrous substrates into volatile fatty acids and microbial protein that are further used by the ruminant for upholding, growth, and lactation [12, 13, 15]. Despite the highly evolved and specific rumen environment, less than 50% of carbohydrates in low-quality forages, such as straw, are digested or utilized by the host [12]. The passage of lignocellulosic biomass through the rumen and limited access of fibrolytic enzymes to their targeted substrates are believed to restrict the degradation extent of plant cell walls in the rumen [16]. Understanding these limitations and the complete mechanism of plant cell wall degradation by rumen microbes is crucial for developing approaches to improve forage utilization in ruminants [12]. Furthermore, reducing enteric methane emissions represents a significant opportunity to decrease global methane emissions [17, 18]. Livestock production and its associated by-products contribute approximately 51% of global warming gases, releasing at least 32.6 billion tons of CO_2 annually [2, 19]. While carbon dioxide is the primary greenhouse gas (GHG (55–60%), methane (15–20%) follows as the second most significant contributor, with livestock being a major source through ruminal fermentation [2, 20]. Understanding methanogenic and methanotrophic species in ruminant livestock through dietary modification can help identify new approaches to reduce greenhouse gases [17, 18]. The impacts of diverse feed components on rumen function have been extensively studied. The incorporation of unsaturated fatty acids (UFA), specifically soybean oil and whole raw soybean, in ruminant diets has shown improved ether extract digestibility while maintaining rumen pH stability, without compromising feed intake and digestion parameters [21]. Research on various roughage sources demonstrated differential effects on nutrient digestibility and rumen fermentation, with whole corn silage exhibiting superior digestibility compared to sugarcane shoot silage which showed the lowest digestibility values [22]. Investigations into oak kernel supplementation revealed increased dry matter intake and digestibility, though protein digestibility decreased, however, rumen fermentation characteristics remained unaffected [23]. Additionally, yeast-fermented cassava pulp supplementation demonstrated enhanced rumen microbial protein synthesis and improved fermentation end-products, leading to better overall nutrient digestibility [24]. These findings emphasize that optimizing the balance of carbohydrates, proteins, fats, and roughage can significantly enhance rumen fermentation, nutrient digestibility, and overall productivity.

Given the complexity of the rumen microbiome, "-omics" based approaches, particularly metagenomics and metatranscriptomics, are widely employed to study their phylogeny and function [12]. Multiple "-omics" based studies have characterized the

lignocellulose-degrading activity of the rumen bacterial community [11, 25]. While these studies provide insights into microbial community composition, they offer limited information about community function, particularly regarding plant cell wall decomposition within the rumen [12]. Recent advances in sequencing technologies and bioinformatics, coupled with reduced sequencing costs, have enabled researchers to perform metagenomics sequencing that can identify complete rumen genes. This approach provides comprehensive information about both the organisms present and their metabolic potential and function within the overall rumen microbial community [12].

Gene signature and biological fingerprinting of rumen microbes represent a crucial area of scientific research [26]. Recent advances in ruminant gut microbiology and genomics have opened new approaches for carrying out comprehensive analyses of rumen ecological structure and function. The importance of rumen microbial signatures and microbial diversity in the ruminant forestomach has gained increasing attention owing to current trends in global livestock production [26]. Metagenomics provides detailed insights into the functional dynamics of the ruminomics database and supports a primary goal of rumen ecosystem research, i.e. understanding microbial community functions and their interaction with both other microbes and the host [26]. While metagenomic approaches typically favor the most abundant genes from predominant microbial species [27], a gene's significance in plant cell wall degradation may not directly correlate with its abundance. In contrast, metatranscriptome profiling captures the composition and relative abundance of actively transcribed genes [25, 27]. Consequently, rumen metatranscriptomic studies provide in-depth insight into the rumen's functional capacity. By comparing rumen metatranscriptome profiles under various conditions, researchers can understand how the rumen microbial community modifies and adapts to environmental changes [12].

While diet significantly influences rumen microbial composition, most research has focused on dairy cows, with limited studies on water buffalo (*Bubalus bubalis*), which typically consumes a mixture of roughages and concentrates [4]. The present study analyzed forty-eight publicly available rumen metatranscriptome samples from Mehsani buffalo subjected to six different diet treatments. The research aims to determine taxonomic and functional variations in buffalo rumen across different diets, with particular emphasis on digestive enzymes involved in carbohydrate transport and metabolism, polysaccharide metabolism, fermentation, and methanogenesis. Furthermore, statistical and computational analysis were conducted to strengthen the findings and establish metabolism's dominant role through a computationally rigorous approach. The study's primary objective was to elucidate the effects of feed variation on carbohydrate metabolism, fermentation, and methanogenesis in the rumen, while understanding the source of this variation at both pathway and gene levels, providing a robust foundation for researchers in the field of rumen feed management and nutrition.

Materials and methods

Metatranscriptome datasets

Current study focuses on forty-eight metatranscriptome datasets of the rumen microbiome of Mehsani Buffalo. These samples were taken from MG-RAST (https://www.mg-rast.org/linkin.cgi?project=mgp14932) [28]. Data was generated from eight healthy, non-lactating, non-pregnant female Mehsana buffaloes (*B. bubalis*) weighing around 450 kg and aged 4–5 years. Three diets were created with roughage to concentrate ratios of 50:50 (1), 75:25 (2), and 100:0 (3). Each diet treatment was fed to 4 buffaloes with green roughage (Sorghum bicolor) and 4 with dry roughage. After 6 weeks of adaptation to the diets, samples were collected 3 h post-feeding and the buffaloes were shifted to the next diet treatment, starting from 1, followed by 2 and then 3 [29]. Thus, resulting in a total of six different diet treatments, i.e., DR (100% dry roughage), GR (100% green roughage), DR5C5 (50% dry roughage and 50% concentrate), GR5C5 (50% green roughage and 50% concentrate), DR7C2 (75% dry roughage and 25% concentrate), and GR7C2 (75% green roughage and 25% concentrate). Eight individual samples were available for each diet treatment, thus resulting in a total of 48 datasets. Details of all these datasets are provided in Table S1.

Analysis of rumen samples

Each dataset was processed for further downstream analysis which was mainly focused on analysis of metabolic activities associated with feed variations. For this purpose, taxonomic and functional annotation followed by analysis of metabolic pathways and digestive enzymes were carried out individually for all datasets. Each sample was processed through the standard pipeline of MG-RAST with default parameters. For taxonomic annotation, RefSeq was taken as a reference database while for functional analysis SEED subsystems, COG, and KEGG Orthology (KO) were taken as reference [28]. Parameters considered were: minimum percentage identity: 60%, maximum E-value: 1×10^{-5}, and minimum alignment length: 15, for both taxonomy and functional annotation. Minimum Percentage Identity at 60% allows to capture moderately divergent but still homologous sequences. This threshold helps

balance sensitivity and specificity in sequence matching. Maximum E-value at 1×10^{-5} is a relatively stringent E-value cutoff that helps control false positives. Keeping minimum alignment length at 15 amino acids/base pairs ensures that matched sequences are long enough to indicate true homology, as 15 residues is considered sufficient to represent a functional domain or motif. Metabolic pathways, their corresponding genes/transcripts, and enzymes were identified for different biological processes, particularly involved in carbohydrate metabolism in each sample from all the diet groups. The results of the taxonomic and functional annotation were subsequently analyzed in Statistical Analysis of Metagenomic Profiles (STAMP) [30].

Identification of prominent pathways associated with feed variation

Functional profile was generated for all datasets using KEGG orthology database as a reference and it was further used for performing different statistical analyses for identifying pathways significantly affected by diet treatments [28]. Statistical analysis of metatranscriptome data was carried out using shotgun data profiling in MicrobiomeAnalyst [31]. Each dataset was processed for various analyses such as principal component analysis, univariate statistical comparisons, pattern search, differential abundance analysis, Linear discriminant analysis Effect Size (LEfSe), and Random Forest analysis. For the low count filter in the dataset, mean abundance value was considered while for low variance filter, the standard deviation was used. As a result, a total of 105 low abundance features (i.e. pathways) were removed on the basis of mean while 15 low variance features were removed based on standard deviation. Only 126 features or pathways were allowed after data filtration. The data normalization was done where, cumulative sum scaling (CSS) was used for data scaling. Univariate statistical comparisons were carried out through T-test/ANOVA with p-value cutoff at 0.05. Pattern search was applied for the identification of pathways correlated with different diet treatments using Kendall rank correlation as a distance measure. Differential abundance analysis was carried out using metagenomeSeq and a zero-inflated Gaussian fit (p-value cut-off 0.05) statistical model was applied. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was done with p-value cutoff of 0.1 and Log LDA score of 2.0. Random forest classification was carried out to identify pathways important for microbiome data classification as per diet treatments. 500 trees were allowed to grow and the number of predictors to try were seven with randomness on, and the plots included the out-of-bag (OOB) error [31].

Results

Assessment of microbial community abundance

Forty-eight rumen metatranscriptome datasets were analyzed, representing a total of 8420.78 Mb data, with individual dataset sizes ranging from 99.10 to 235.94 Mb and read counts varying from 610,377 to 1,15,1786 (Table S1). Taxonomic annotation at the domain level revealed that bacteria constituted the most abundant microbial community across most datasets, followed by Eukaryota, archaea, and viruses (Fig. 1).

Prevotella emerged as the predominant microbial community across the majority of the dataset, maintaining a significant presence across all diet treatments (Fig. 2). In samples with green roughage, while *Prevotella* remained the most abundant genus, its mean distribution in GR and GR5C5 was lower than in DR and DR5C5. However, in the 75% roughage and 25% concentrate treatment, GR7C2 showed higher mean distribution than DR7C2 (Fig. 2). Bacteroides, Clostridium, and Firmicutes also maintained significant presence (Fig. S1), consistent with previous findings [17, 18]. Analysis of methanobacteria abundance across diet groups revealed a wide distribution of methanogens in diet treatment with dry roughage (Fig. S1). At the species level, *Prevotella ruminicola* dominated most datasets, with some notable exceptions. In DR, *P. ruminicola* was the most abundant species across all eight datasets. In DR5C5, three datasets (M1DL1, M1DS3, and M1DS4) showed *Emericella nidulans, Ruminococcus obeum,* and *Nematostella vectensis*, as the most abundant species, respectively. The DR7C2 diet group showed, *Prevotella ruminicola* dominance except in two samples, M2DL4 (*Hyaloraphidium curvatum*) and M2DS4 (*Barssia oregonensis*). In the GR group, *Gallus gallus* dominated four datasets (M3GS1-M3GS4) while for the rest datasets, *Prevotella ruminicola* was abundant one. The GR5C5 treatment showed *Prevotella ruminicola* dominance except in M1GS2 (*Nematostella vectensis*) and M1GS4 (*Physoderma maydis*), while *Prevotella ruminicola* Dominated consistently across all GR7C2 samples. From the above results, it is clear that *Prevotella ruminicola* occupied significant distribution across all diet treatments.

Analysis of functional profile of datasets

Functional profile identification and annotation, conducted using COG and SEED subsystems as reference databases, revealed the predominance of metabolic processes compared to other biological processes (Fig. 3). Diet composition significantly influenced the functional profile, with metabolism being most abundant in green

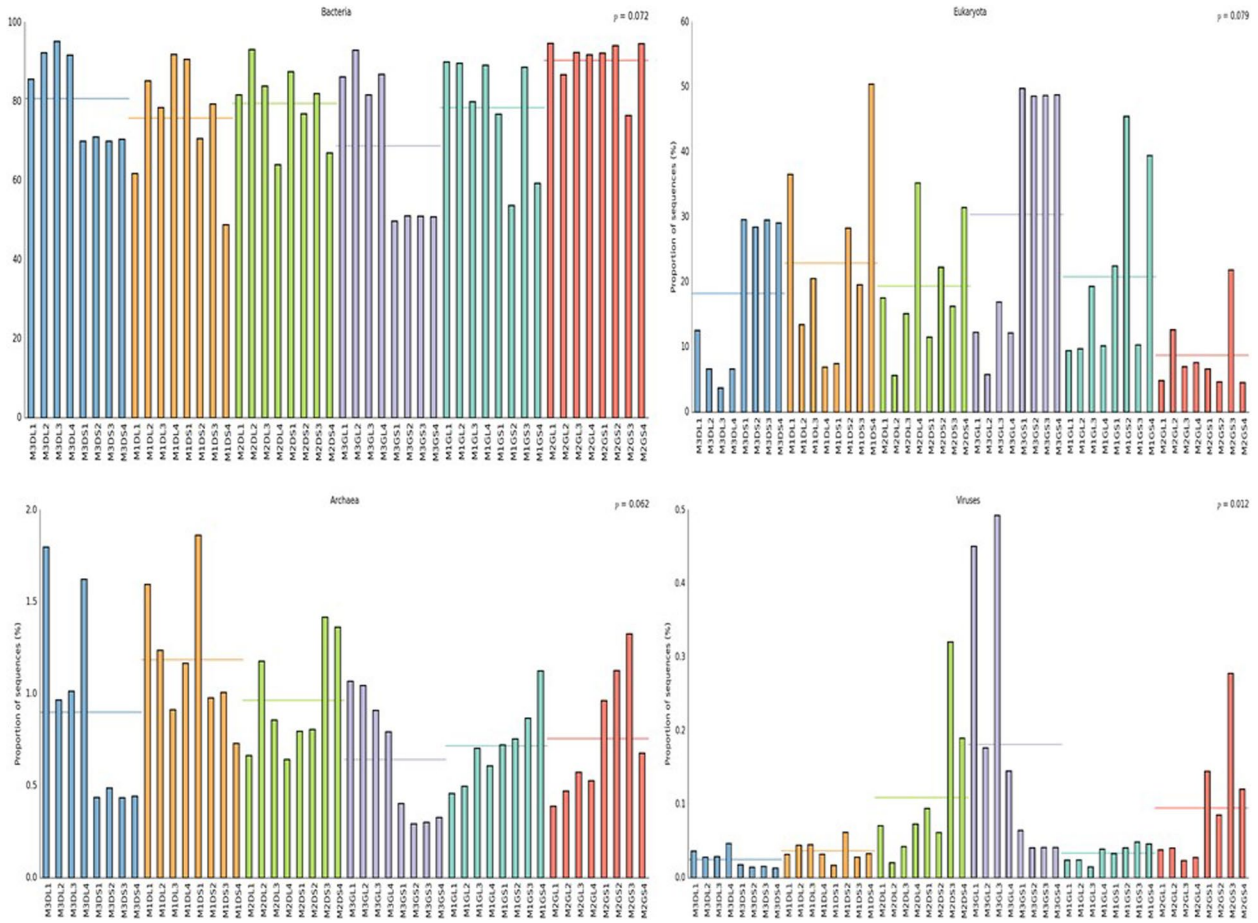

Fig. 1 Taxonomic abundance in forty-eight rumen metatranscriptome samples at domain level, i.e. Bacteria, Eukaryata, Archea and Viruses. Color coding varies as DR, DR5C5, DR7C2, GR, GR5C5, GR7C2. The X-axis shows the name of each dataset whereas the y-axis represents the proportion of sequences. The value of y-axis is different for all 04 domains, it varies as, Bacteria: 0–100, Eukaryota: 0–60, Archea: 0–2, and Viruses: 0–0.5

roughage (GR) treatments and its combinations with concentrate (GR5C5 and GR7C2) (Fig. 3). In contrast, dry roughage (DR) treatments showed high abundance of both Metabolism and Information Storage and Processing, a pattern also observed in DR7C2. Notably, when concentrate and dry roughage were provided in equal proportion (DR5C5), metabolism emerged as the dominant biological process (Fig. 3), suggesting that roughage type significantly influences rumen metabolic activities. This metabolic dominance aligns with findings from beef cattle rumen microbiome [32].

Further analysis of metabolic activities revealed eight distinct pathways, Amino acid transport and metabolism (AATM), Carbohydrate transport and metabolism (CTM), Coenzyme transport and metabolism (CoTM), Energy production and conversion (EPC), Inorganic ion transport and metabolism (IiTM), Lipid transport and metabolism (LTM), Nucleotide transport and metabolism (NTM), Secondary metabolites biosynthesis, transport and catabolism (SMBT) (Fig. 4). Among these, CTM demonstrated the highest prevalence, followed by EPC and AATM (Fig. 4). LTM, NTM and CoTM also showed their significant presence (Fig. 4). Analysis identified 1066 different enzyme types across these pathways, with carbohydrate and amino acid metabolism showing particular predominance in green roughage diet treatments (Fig. 4).

Using the SEED subsystem database as a reference, analysis of metabolic capacities associated with carbohydrate metabolism revealed higher activity in green roughage and its associated diet groups compared to dry roughage. Twelve different metabolic processes were identified in the rumen metatranscriptome of mehsani buffalo for carbohydrates metabolism, i.e., monosaccharides, central carbohydrate metabolism, fermentation, polysaccharides, one-carbon metabolism, di- and oligo-saccharides, CO_2 fixation, organic acids, glycoside hydrolases, amino sugars, sugar alcohols and others/

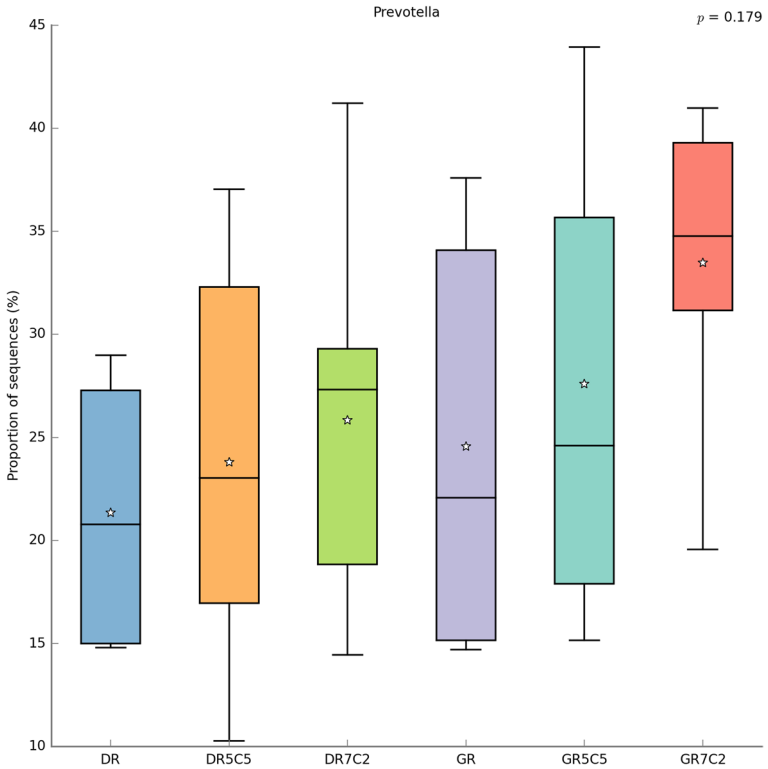

Fig. 2 Distribution of microbial community '*Prevotella*' and variation in its distribution. The X-axis shows the name of meal groups whereas the y-axis represents the proportion of sequences

miscellaneous. Central carbohydrate metabolism was most prominent, followed by mono-saccharides, one-carbon metabolism, and di- and oligo-sacchrides metabolic activities (Table S2). One-carbon metabolism encompasses genes involved in the serine-glyoxylate cycle while the Di- and Oligo-saccharide category includes genes associated with various sugar utilization pathways, including maltose/maltodextrin utilization, l-rhamnose utilization, xylose utilization, l-arabinose utilization, lactose utilization, mannose utilization [17, 18].

An in-depth analysis of central carbohydrate metabolism revealed seventeen distinct pathways, viz., Dehydrogenase complexes, Dihydroxyacetone kinases, Entner-Doudoroff Pathway, Ethylmalonyl-CoA pathway of C2 assimilation, Glycolate, glyoxylate interconversions, Glycolysis and Gluconeogenesis, including Archaeal enzymes, Glyoxylate bypass, HPr kinase and hprK operon in Gram-positive organisms, Methylglyoxal Metabolism, Pentose phosphate pathway, Peripheral Glucose Catabolism Pathways, Pyruvate: ferredoxin oxidoreductase, Pyruvate Alanine Serine Interconversions, Pyruvate metabolism I: anaplerotic reactions, PEP, Pyruvate metabolism II: acetyl-CoA, acetogenesis from pyruvate and TCA Cycle. The number of enzymes identified varied across diet groups, i.e., 99 (GR), 108 (DR), 96 (GR7C2), 103 (DR7C2), 105(GR5C5) and 98 (DR5C5). Some enzymes were exclusively present in specific diet treatments, ie., 15 (GR), 11 (GR5C5), 5(GR7C2), 14 (DR), 6 (DR5C5) and 10 (DR7C2).

Fermentation activities in rumen metatranscriptome

Ruminant feed conversion efficiency depends not only on the animal's genetic potential to absorb and utilize nutrients but also on the rumen microbiota's ability to ferment diet components into volatile organic acids [33]. Analysis of fermentation activity revealed similar levels between dry and green roughage treatments when no concentrate was present or when given in equal amounts (DR vs GR and DR5C5 vs GR5C5) (Fig. 5). However, with 25% concentrate (DR7C2 vs GR7C2), green roughage feed demonstrated higher fermentation activity (Fig. 5). Six fermentation associated pathways were identified, i.e., Acetoin, butanediol metabolism, Acetone Butanol Ethanol Synthesis, Acetyl-CoA fermentation to Butyrate, Butanol Biosynthesis, Fermentations: Lactate and Fermentations: Mixed acid. Each diet treatment showed varying numbers of enzymes, 31 (GR), 34 (DR), 36 (GR7C2), 34 (DR7C2), 32(GR5C5), and 34 (DR5C5). Certain enzymes were present only in 01 treatments, i.e., GR (3-hydroxybutyrate dehydrogenase (EC 1.1.1.30)),

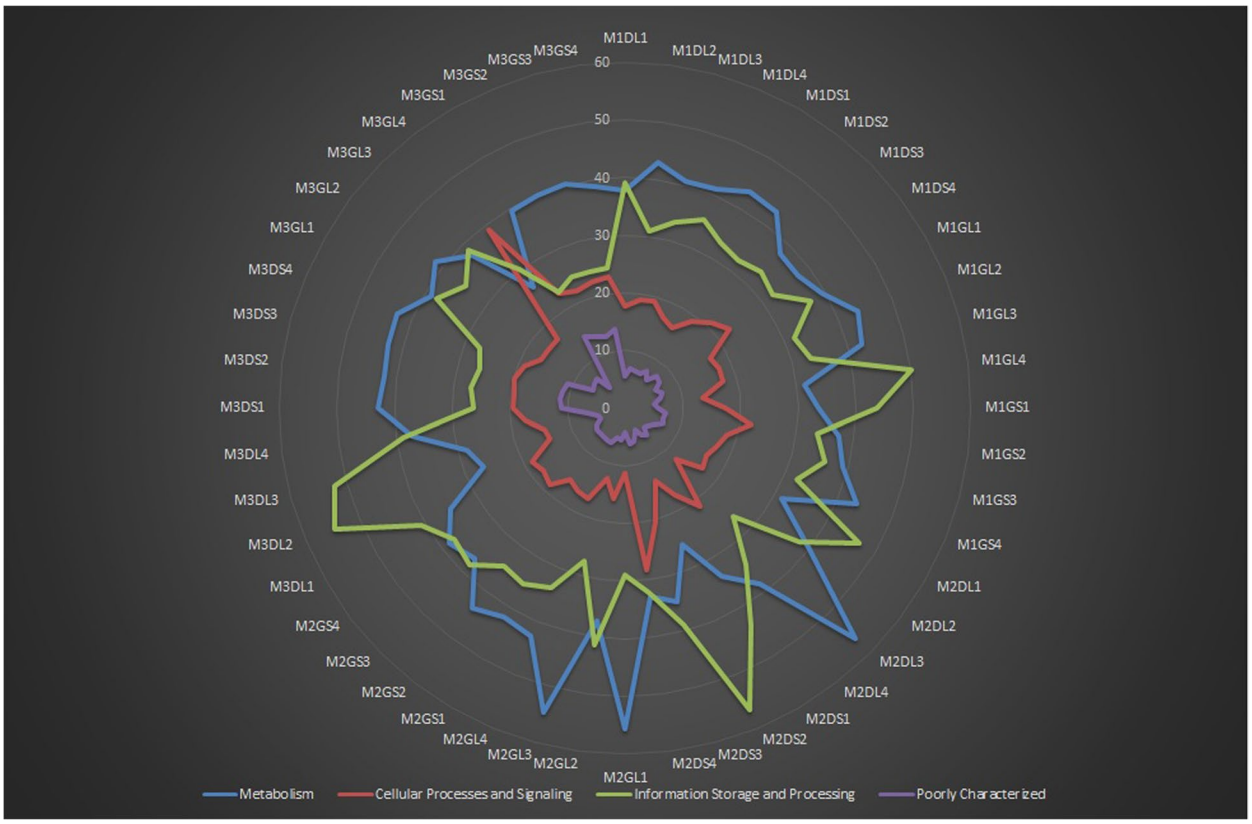

Fig. 3 Functional profile of rumen metatranscriptome samples taking COG as reference database. DR5C5: M1DL1-M1DL4, M1DS1-M1DS4; GR5C5: M1GL1-M1GL4, M1GS1-M1GS4; DR7C2: M2DL1-M2DL4, M2DS1-M2DS4; GR7C2: M2GL1-M2GL4, M2GS1-M2GS4; DR: M3DL1-M3DL4, M3DS1-M3DS4; GR: M3GL1-M3GL4, M3GS1-M3GS4

DR (Acetoacetate metabolism regulatory protein AtoC, Transcriptional activator of acetoin dehydrogenase operon AcoR), GR5C5 (Acetolactate synthase, catabolic (EC 2.2.1.6), Butyryl-CoA dehydrogenase (EC 1.3.99.2), Phosphoenolpyruvate carboxylase (EC 4.1.1.31)), DR7C2 (Acetoin catabolism protein X, Ferredoxin-like protein). For GR7C2 and DR5C5, no unique gene was identified.

Enzyme 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) which was present only in GR diet, is involved in the synthesis and degradation of ketone bodies and the metabolism of butyric acid. Acetolactate synthase, catabolic (EC 2.2.1.6), catalyzes first step in synthesis of the branched-chain amino acids (valine, leucine, and isoleucine) while Phosphoenolpyruvate carboxylase (EC 4.1.1.31), converts oxaloacetate in the tricarboxylic acid cycle when operating in the reverse direction and both of these enzymes were present in GR5C5 only. Acetate, propionate, and butyrate stand out as the volatile fatty acids linked to efficient animal feed. Acetate and butyrate contribute to fat production and energy supply, while propionate is the primary supplier of glucose to ruminants [34, 35].

Methanogenesis and methane metabolism in rumen metatranscriptome

The study of metatranscriptome datasets for methanogenesis can help in providing the role of diet variations in this process. Methanogenesis was analyzed in all of these samples using SEED subsystems as a reference database. Different enzymes associated with methanogenesis activity were identified, i.e., 31 (GR), 36 (DR), 32 (GR7C2), 35 (DR7C2), 32(GR5C5) and 35 (DR5C5) (Fig. 5). Diet treatments with dry roughage showed higher methanogenesis compared to green roughage treatments (Fig. 5). Three of the diet treatments possess some enzymes which were absent from rest of the five treatments, i.e., DR7C2 (Methyl coenzyme M reductase associated protein), GR7C2 (Formylmethanofuran dehydrogenase (molybdenum) subunit C (EC 1.2.99.5), N5-methyltetrahydromethanopterin: coenzyme M methyltransferase subunit H (EC 2.1.1.86)), and DR (Formylmethanofuran dehydrogenase (tungsten) subunit B (EC 1.2.99.5), Formylmethanofuran dehydrogenase subunit C (EC 1.2.99.5)). While for GR, GR5C5, DR5C5, no unique methogenesis enzyme was identified. It was clear from

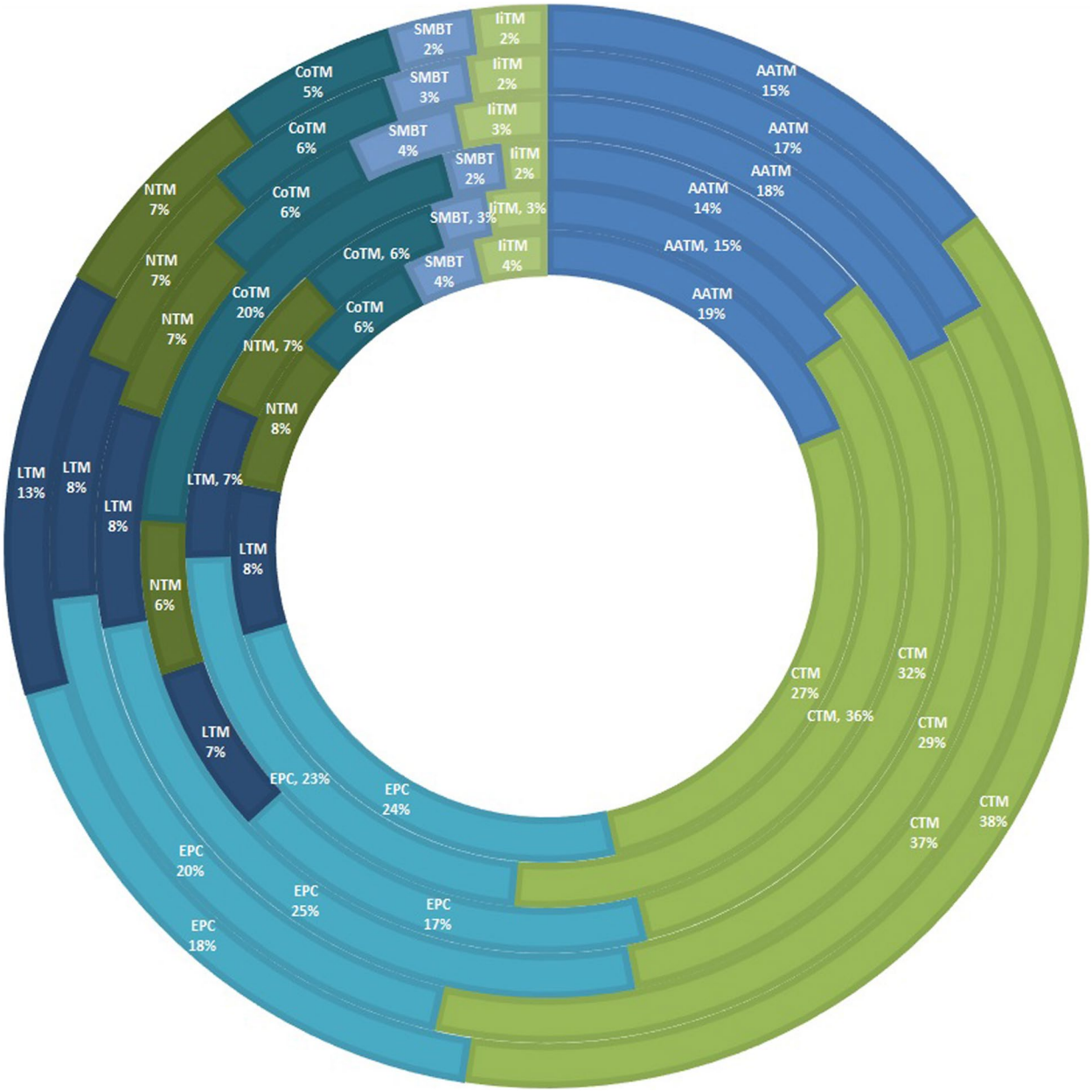

Fig. 4 Percentage distribution of eight different metabolic pathways in rumen metatranscriptome. Inner most circle represents DR, followed by DR5C5, DR7C2, GR, GR5C5 and GR7C2 respectively. Abbreviations used are Amino acid transport and metabolism: AATM; Carbohydrate transport and metabolism: CTM, Coenzyme transport and metabolism: CoTM, Energy production and conversion: EPC, Inorganic ion transport and metabolism: IiTM, Lipid transport and metabolism: LTM, Nucleotide transport and metabolism: NTM, Secondary metabolites biosynthesis, transport and catabolism: SMBT

the above observations that roughage along with concentrate proportion influences methanogenesis.

Pathway mapping for methane metabolism (KO00680) identified several abundant enzymes across the datasets (Fig. S2). A number of different enzymes were identified for the same. Few enzymes which were abundantly present across the datasets were ppdK; pyruvate, orthophosphate dikinase [EC:2.7.9.1] (K01006), TUBB; tubulin beta (K07375), TUBA; tubulin alpha (K07374), fliC; flagellin (K02406), tuf, TUFM; elongation factor Tu (K02358), ENO, eno; enolase [EC:4.2.1.11] (K01689), EEF1A; elongation factor 1-alpha (K03231), E2.4.1.1, glgP, PYG; starch phosphorylase [EC:2.4.1.1] (K00688), rpoB; DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]

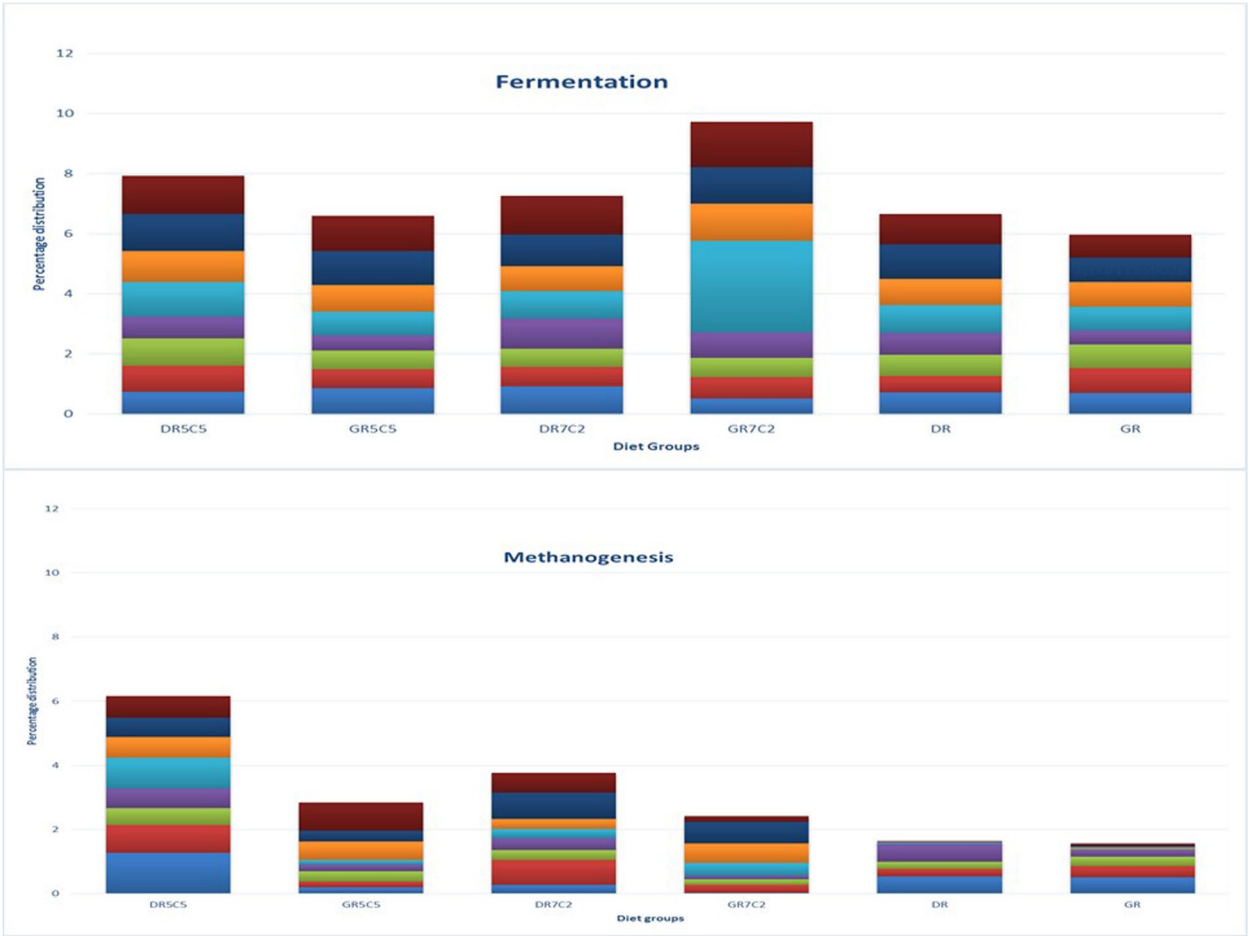

Fig. 5 Observed fermentation and methanogenesis activity in rumen metatranscriptome of different feed variations

(K03043), htpG, HSP90A; molecular chaperone HtpG (K04079), HSPA1_8; heat shock 70 kDa protein 1/8 (K03283), and rpoC; DNA-directed RNA polymerase subunit beta' [EC:2.7.7.6] (K03046) (Fig. S2). Tuf, TUFM; elongation factor Tu (K02358) were the most abundant enzymes across all the datasets.

Analysis of metatranscriptome functional profiles for identification of pathways associated with feed variation

To better understand functional profile variation among different diet treatments, comprehensive analysis were conducted. Principal component analysis revealed distinct clustering patterns, with roughage-only samples (both dry and green) forming seperate clusters from other diet treatments (Fig. S3). Clustering was also carried out for these samples using Ward clustering algorithm and Bray–Curtis index as distance measure to get a better overview of between-group variation and within-group variation (Fig S3). Univariate statistical comparisons using T-test/ANOVA identified 64 significant pathways related to diet variations, with the majority (twenty) associated with metabolic processes. These pathways were involved in Amino acid metabolism (KO00290, KO00300, KO00310, KO00360, KO00400), Carbohydrate metabolism (KO00500, KO00620, KO00630), Metabolism of cofactors and vitamins (KO00130, KO00730, KO00860), Metabolism of other amino acids (KO00440, KO00480), Biosynthesis of other secondary metabolites (KO00521, KO00940), Energy metabolism (KO00195), Glycan biosynthesis and metabolism (KO00510), Metabolism of terpenoids and polyketides (KO00908), Nucleotide metabolism (KO00230) and Xenobiotics biodegradation and metabolism (KO00983), highlighting amino acid metabolism as the predominant metabolic category in ANOVA analysis.

To further strengthen the observed results and establish the dominant role of metabolism via a computationally aware method, pattern search analysis was applied from microbiome analysts to identify pathways correlated with different diet treatments, and the top 25 pathways were identified which correlated with the diet treatments. The

pathways were ranked by their correlation. Thirteen pathways showed positive correlation, while twelve showed negative correlation (Fig. 6). Among positively correlated pathways, all except three were associated with metabolism (KO04112: Cellular Processes, Cell growth, and death, Cell cycle – Caulobacter; KO02060: Environmental Information Processing, Membrane transport, Phosphotransferase system (PTS); KO05132: Human Diseases, Infectious diseases, Salmonella infection). Three were associated with amino acid metabolism (KO00280 and KO00290: Valine, leucine, and isoleucine biosynthesis; KO00360: Phenylalanine metabolism). For carbohydrate metabolism and metabolism of cofactors and vitamins, two pathways were identified, i.e. KO00010 (Glycolysis/Gluconeogenesis) and KO00040 (Pentose and glucuronate interconversions). In the case of metabolism of cofactors and vitamins, KO00130 (Ubiquinone and other terpenoid-quinone biosynthesis) and KO00790 (Folate biosynthesis) were identified. The rest of KO terms were associated with Energy metabolism (KO00195), Glycan biosynthesis and metabolism (KO00550), and Metabolism of terpenoids and polyketides (KO00900) (Fig. 6). Out of twelve pathways with negative correlation, none were associated with metabolism. They were from different groups, i.e., Cellular Processes (KO04810, KO04520, KO04510, KO04145), Environmental Information Processing (KO04310, KO04151, KO04080), Genetic Information Processing (KO04141), Human Diseases (KO05200, KO05143) and Organismal Systems (KO04972, KO04672) (Fig. 6). Further, it can be observed that positively correlated pathways were showing more intense distribution in GR5C5 and GR7C2 with few exceptions (Fig. 6). Above observations clearly reflect that metabolism is most prominently affected biological process by diet variations.

Differential abundance analysis for identification of significantly abundant pathways

Differential abundance analysis was carried out to identify significantly abundant pathways using metagenomeSeq (https://github.com/HCBravoLab/metagenomeSeq). metagenomeSeq is able to identify features that are differentially abundant between two or more groups of multiple samples. A total of 50 significant pathways were identified, where 17 pathways were associated with metabolism (Table S3). Differential analysis of these metabolic pathways across diet treatment revealed that green roughage groups, particularly when supplemented with concentrate (50% or 25%), showed higher abundance of metabolic pathways compared to dry roughage treatments (Fig. S4).

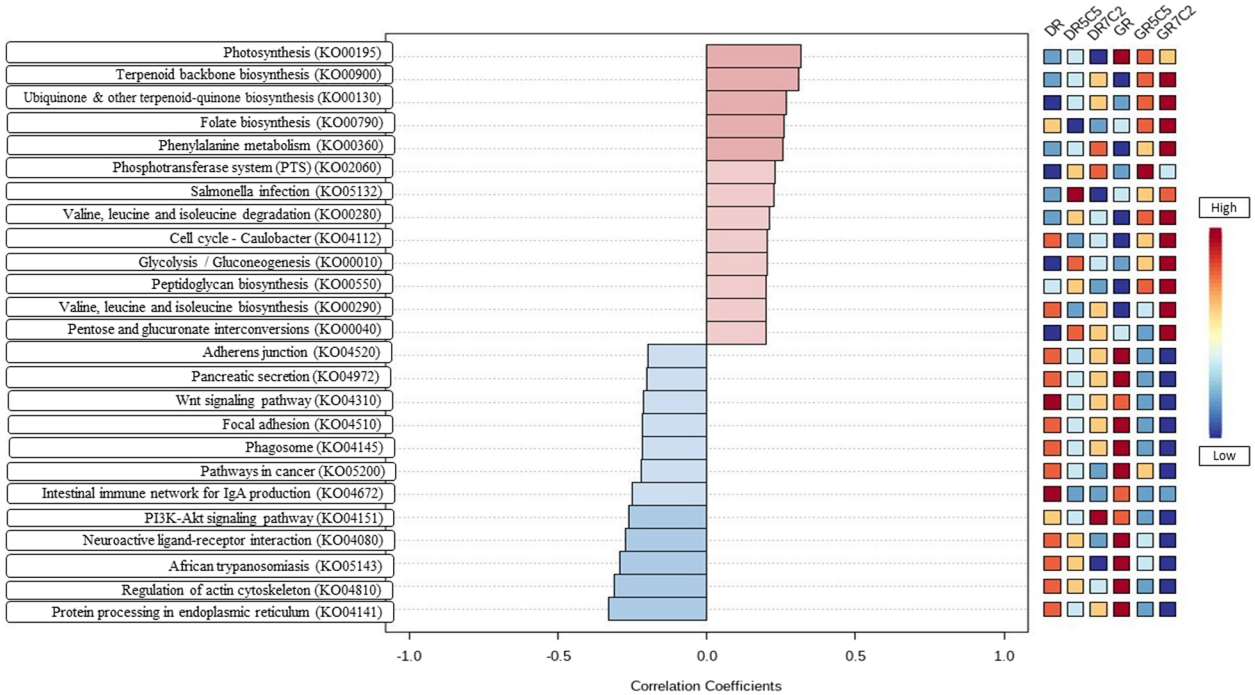

Fig. 6 Pattern search with Kendall rank correlation as distance measure was applied to identify pathways correlated with all the six diet treatments. Correlation coefficients are depicted as positive (red) or negative correlations (blue). On the right mini heatmap is given which denotes the abundance of corresponding pathways in diet types

Biomarker analysis

Linear discriminant analysis (LDA) effect size (LEfSe) analysis was employed to identify significant differences among feed variations. The analysis revealed 10 significant pathways responding to diet treatments (Fig. S5), distributed across various biological processes i.e., Metabolism (KO00900, KO00620, KO00500, and KO00230), Cellular Processes (KO04540 and KO02040), Environmental Information Processing (KO04151 and KO02010) and Genetic Information Processing (KO03020 and KO03010). The results confirmed metabolism as the dominant and most significant biological process in the rumen metatranscriptome samples. Among the four metabolic pathways identified, two were associated with Carbohydrate metabolism (Pyruvate metabolism (KO00620) and Starch and sucrose metabolism (KO00500)) while single pathway was identified for Nucleotide metabolism (Purine metabolism: KO00230) and Metabolism of terpenoids and polyketides (Terpenoid backbone biosynthesis: KO00900) each. Analysis by diet treatment revealed distinct patterns. In the dry roughage-only diet (DR), pathways associated with Genetic Information Processing (KO03020 and KO03010) emerged as most significant. DR5C5 showed significance in pathways related to Cellular Processes and Environmental Information Processing (KO04540, KO02040 and KO02010) while DR7C2 showed KO00500 and KO04151 as significant ones, where KO0050 was associated with metabolism (Fig. S5). When green roughage and concentrate were provided in equal proportions (GR5C5), KO00620 emerged as the most significant pathway which corresponds to Pyruvate metabolism (Fig. S5). The GR7C2 treatment showed significance in metabolic pathways, specifically Terpenoid backbone biosynthesis (KO00900) and Purine metabolism (KO00230) (Fig. S5). The biomarker analysis confirmed metabolism as the predominant biological process in buffalo rumen, with green roughage demonstrating more pronounced metabolic activity compared to dry roughage.

Random forest analysis

Random forests classification (Out of Bag (OOB) error = 0.375) further supported the central role of metabolism, with four of the 15 differentiating factors belonging to metabolic pathways, – Photosynthesis (KO00195),—Lysine degradation (KO00310),—Phosphonate and phosphinate metabolism (KO00440) and -Pyruvate metabolism (KO00620) (Fig. S6). The random forest analysis was evaluated based on the global prediction error rate after 500 random forests.

Discussion

Ruminal microorganisms play a crucial role in providing energy to their hosts [33, 36]. While numerous studies have investigated the relationship between the rumen microbiome and feed efficiency in cattle, feed efficiency remains a complex trait involving multiple host biological processes [33, 36, 37]. Such assosciations have been demonstrated in both dairy cows [33, 36, 38–40] and beef cattle [33, 41–47]. Functional analysis of rumen microbiota suggests that differences between high and low feed efficiency animals may be attributed to genes associated with carbohydrate digestion (fibrous and non-fibrous), fatty acid and protein synthesis, energy conservation pathways (such as ATP production), and methane production [33, 38].

The remarkable capacity of ruminants to digest plant polysaccharides stem from their complex rumen microflora [1]. The rumen has evolved as an efficient fermentation chamber for fiber degradation, hosting a diverse microbial community including bacteria, archaea, viruses, fungi, and protozoa [1, 6, 7]. Bacteria dominate this community, comprising approximately 95% of total microorganisms [1, 48, 49]. The microbially-governed rumen fermentation process influences milk and meat quality and composition, as well as host productivity [1, 50–52]. Fibrolytic rumen microbes have developed specialized structures like "cellulosomes" and multifunctional enzymes capable of hydrolyzing diverse fibrous substrates [12, 53].

In our study, the identification of *Prevotella* as the dominant microbial community (Fig. 2) aligns with previous findings from Mehsani buffalo metagenome studies [17, 18] and goat rumen microbiome research [54]. *Prevotella* is known for volatile fatty acid production, particularly propanoate, which serves as an energy source for the host [55, 56], and plays a significant role in carbohydrate and nitrogen metabolism [57]. Previous research has shown that *Prevotella* population increases when methanogenesis (a hydrogen-consuming process) is inhibited [58, 59], suggesting that enhanced *Prevotella* abundance may contribute to reduced methanogenesis [55, 60–62]. *P. ruminicola* possesses diverse glycoside hydrolases targeting non-cellulosic polysaccharides, particularly GH43 bifunctional enzymes [63], and demonstrates capability in pectin degradation [64]. Prevotella is a predominant genus in the rumen microbiome, playing a crucial role in nutrient breakdown and metabolism, particularly of complex carbohydrates and proteins [57, 65]. Its interactions with other microbial communities in the rumen significantly influence the overall functionality and health of the host. Notably, Prevotella and Ruminococcus genera demonstrate a strong co-exclusion relationship, forming distinct microbial clusters within the rumen, similar

to the enterotype-like clustering observed in pig microbiota [66]. Prevotella establishes synergistic relationships with methanogenic archaea through mechanisms such as interspecies hydrogen transfer, which helps maintain the balance of volatile fatty acids (VFAs) and other metabolic byproducts in the rumen [67, 68]. The abundance of Prevotella influences VFA production, critical for the host's energy supply, while variations in its population can significantly affect rumen pH and methane production [68, 69]. Studies have linked Prevotella abundance to feed efficiency in ruminants, with efficient animals exhibiting distinct microbial profiles compared to less efficient ones [42]. Dysbiosis resulting from imbalances in Prevotella populations has been associated with various ruminant health issues [70, 71]. Prevotella's interactions with other microbial communities in the rumen are complex and have significant implications for the host's nutrition, health, and productivity. Further research into these interactions can provide deeper insights into optimizing ruminant health and production. The significant abundance of Bacteroides, Clostridium, and Firmicutes observed in this study (Fig. S1) aligns with previous metagenome findings from similar feed variation [17, 18]. The Bacteroides group relies on soluble polysaccharides produced by other bacteria in the rumen's liquid phase [55, 72, 73]. Both Bacteroidetes and Firmicutes population in the rumen are associated with organic matter conversion to simpler forms [55, 74, 75]. Bacteroidetes, commonly found in both human gastrointestinal tracts and rumens [17, 18], play a crucial rolein protein and carbohydrate degradation [17, 18, 73].

Functional annotation analysis revealed that 'Carbohydrate transport and metabolism' was more prominent in green roughage diets compared to dry roughage (Fig. 3; Table S2). Previous studies have also documented the abundance of metabolism-related functional categories, particularly carbohydrate, amino acid, and energy metabolism in the rumen microbiome [14, 32]. The predominance of carbohydrate metabolism in mehsani buffalo has been previously reported [17, 18]. High-milk protein yield cows shows significant *Prevotella* abundance, which enhances functions related to branched-chain amino acid biosynthesis [76]. Rumen metaproteome study in cows have identified carbohydrate metabolism, nucleotide metabolism, pyruvate metabolism, and amino acid metabolism as the most abundant protein families [77]. Similar findings regarding the abundance of genes associated with carbohydrate utilization and metabolism have been reported in other bovine and cow rumen studies [11, 78]. The current study's identification of glycolysis, electron transport, and carbohydrate metabolism as predominant pathways (Fig. 3, 4, Table S2) aligns with previous rumen meta-proteome findings [77]. Bovine rumen is reported to synthesize the functional genes linked with carbohydrate utilization [17, 18]. One-carbon metabolism involves genes engaged in serine-glyoxylate cycle while the category Di- and Oligo-saccharide possess genes associated with maltose/maltodextrin utilization, l-rhamnose utilization, xylose utilization, l-arabinose utilization, lactose utilization, mannose utilization. [17, 18].

Analysis of Carbohydrate transport and metabolism pathways revealed varying numbers of enzymes across diet treatments, i.e., GR (161), DR (167), GR7C2 (169), DR7C2 (169), GR5C5 (176), and DR5C5 (164) (Fig. 4). Green roughage diet groups demonstrated diverse metabolism and possesses exclusive genes absent in dry roughage treatments. For instance, GR uniquely possessed 2-phosphoglycerate kinase and ABC-type ribose transport system, auxiliary component, while GR5C5 exhibited exclusive enzymes such as 2-phosphoglycerate kinase, ADP-glucose pyrophosphorylase, Beta-N-acetylglucosaminidase, Exo-beta-1,3-glucanase, Fucose dissimilation pathway protein FucU, Glucoamylase and related glycosyl hydrolases. GR7C2 showed unique presence of 2-keto-3-deoxy-6-phosphogluconate aldolase, Fructose-2,6-bisphosphatase and Glucose/sorbosone dehydrogenases. Notably, 2-phosphoglycerate kinase enzyme was exclusively present in all three green roughage dietary groups. This enzyme, reported in various methanogens [79], phosphorylates 2-phosphoglycerate in 2,3-diphosphoglycerate [80]. Glucose dehydrogenase, specific to dry roughage samples, participates in the pentose phosphate pathway and catalyzes D-glucose conversion to D-glucono-1,4-lactone [81]. The enzyme 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase, present only in dry roughage dietary groups (DR, DR5C5, and DR7C2), plays a role in the phenylacetate catabolic process and performs the final step unique to the 4-hydroxyphenylacetic acid catabolism pathway in which 2,4-dihydroxyhept-2-ene-1,7-dioic acid is broken into pyruvate and succinate-semialdehyde [82].

In the rumen, continuous fermentation is sustained by a diverse and complex microbiome. The primary fermentation products, volatile fatty acids (VFA) and microbial crude protein (MCP), supply a substantial portion of the host's energy and protein requirements, regardless of dietary substrates [60]. The rumen microbiome synthesizes various volatile fatty acids, e.g., propionic acid, butyric acid, and acetate through microbial fermentation, which are crucial for maintaining animal health and homeostasis. These VFAs are subsequently utilized for milk and meat production, contributing significantly to the daily energy requirement of ruminants [55].

Methanogenesis, an anaerobic respiratory process yielding methane as its final metabolic product, possesses

unique charateristics despite its theoretical similarity to other respiratory processes. These include notably low energy yield and its restrictions to methanogens, organisms specifically capable of biological methane production [83]. Methane production has become a global concern due to its role as a greenhouse gas [84]. While methane emissions originate from various sources including wetlands, energy sectors, biomass burning, landfills, and ruminants [85], enteric fermentation represents a major contributor [86]. Studying rumen archaeal metabolism to mitigate methane production may help develop strategies to reduce greenhouse effect [87]. Previous studies have shown varying relationships between diet and methane production. In Gir cattle, increased roughage-rich diet led to decreased methane production [55]. A rumen meta-proteome study in cows revealed that high-milk protein yield individuals showed lower relative abundances of methanogen and methanogenesis functions, suggesting reduced methane production [76]. Additionally, metagenomics analysis of Mehsani buffalo demonstrated that animals fed with a combination of roughage and concentrate exhibited lower methanogenesis activity compared to those fed solely with roughage, thus, reflecting the relationship between diet changes and the presence of methanogens and methanotrophs [17, 18]. The current study correlates with these findings, showing that green roughage diet groups demonstrate comparatively lower methanogenesis compared to dry roughage diet treatments (Fig. 5).

Further analysis of the metatranscriptome datasets functional profiles using pattern search, differential abundance analysis, biomarker analysis, and random forest analysis revealed metabolism as the predominant biological process, with significant representation of metabolic pathways, particularly those involved in amino acid and carbohydrate metabolism. Green roughage diet treatments demonstrated substantial metabolic impact, especially when combined with concentrate. The study identified enhanced metabolic activities in the rumens with green roughage, where metabolism emerged as the dominant functional category, while dry roughage diet treatments showed significant distribution across other biological processes as well (Fig. S4, S5). This pattern extended to crucial metabolic processes in the rumen, specifically carbohydrate metabolism and fermentation activity. Additionally, methanogenesis showed higher prevalence in dry roughage diet treatments compared to green roughage diet treatments (Fig. 6, Table S3).

The feasibility of alternate feeds for ruminants depends on multiple factors, including their nutritional value, impact on animal production, and cost-effectiveness compared to conventional feeds. Additional considerations include the environmental impact of both feed production and animal production, as well as the potential economic value of these feeds for alternative purposes such as energy generation [88]. Forage plays a fundamentale role in ruminant nutrition by providing essential fiber. Through the unique anatomical features of ruminants and their symbiotic relationships with rumen microorganisms, forages undergo fermentation to produce volatile fatty acids (VFA), which serve as crucial nutrients for the host animal [89–91]. The significance of forages in ruminant dietary health is well established [91], with forage content directly influencing milk fat synthesis [91, 92]. Research in cattle yak has shown that a higher concentrate-to-forage ratio (70:30) enhances growth performance [93], while studies in Angus cows demonstrate that varying forage-to-concentrate ratios significantly affect growth performance, rumen fermentation, and blood parameters [94]. Therefore, optimizing feed formulation, particularly the forage component, is essential for maximizing dairy production efficiency [91, 95].

Conclusion

In the context of meeting global food security needs in the coming decades, improving feed efficiency is crucial. The current study analyzed forty-eight metatranscriptome datasets from mehsani buffalo across six different diet groups and enhances our understanding of how diet influences Mehsani buffalo rumen microbial ecology, providing valuable insights into the relationship between microbial metabolism and host physiology. The results demonstrate that both forage type (dry or green) and concentrate levels influence the rumen's functional capacity. Metabolic activities were particularly prominent in datasets from green roughage diet treatments, with varying concentrate levels showing distict effects on metabolic capacity. Notably, green roughage diets exhibited reduced methanogenesis activity, suggesting potential environmental benefits through decreased greenhouse gas emissions. The study reveals Prevotella's dominance across all diet treatments, highlighting its significant role in volatile fatty acid production and carbohydrate metabolism. This study enhances our understanding of how diet influences Mehsani buffalo rumen microbial ecology, providing valuable insights into the relationship between microbial metabolism and host physiology. These findings carry significant implications for sustainable livestock farming through multiple pathways. First, the enhanced metabolic activity observed in green roughage-concentrate combinations presents opportunities for optimizing feed formulations, potentially improving feed efficiency while reducing production costs. Second, the lower methanogenesis in green roughage diets offers a promising avenue for reducing greenhouse gas emissions

from livestock farming, aligning with global climate change mitigation efforts. Third, the enhanced understanding of diet composition's relationship with rumen microbial activity enables more informed feed management decisions, potentially improving operational efficiency. A deeper comprehension of rumen microbiology, ecology, and functional capacity, along with the role of feed variation, can lead to improved livestock nutrient utilization efficiency. Further studies investigating feed combinations' effects on rumen microbiome stability and productivity, exploration of metabolic engineering possibilities for optimizing beneficial pathways while reducing methane production, development of novel feed alternatives balancing nutritional value with environmental sustainability, integration of advanced molecular techniques with traditional feeding practices, and comprehensive economic analyses of various feeding strategies can substantially advance our understanding of rumen biology while offering practical insights for developing sustainable livestock farming practices. This could eventually result in enhanced agricultural yields while reducing environmental impact through better control of methane gas emissions.

Supplementary Information

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Supplementary Material 1.

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Declaration of generative AI and AI-assisted technologies in the writing process
During the preparation of this work the author(s) used claude.ai to rearrange the content. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.
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Authors' contributions
RP, RK, SK, AR, JB: Writing-original draft, formal analysis. DCM: Writing, supervision, Conceptualization. UC, MKS: Review & Editing.

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