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Moringa leaf meal exerts growth benefits in small ruminants through modulating the gastrointestinal microbiome

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Abstract

This study investigated the impact of feeding 17% moringa leaf meal (MLM) on the ruminal and fecal microbial composition and body weight gain (BWG) performance of lambs (*Ovis aries*) and kids (*Capra hircus*). A total of $n = 28$ lambs ($n = 14$, no-moringa, $n = 14$, 17% moringa) and 24 kids ($n = 12$, no-moringa, $n = 12$, 17% moringa) were involved in the experiment and body weight was recorded fortnightly. Metagenomic shotgun sequencing was performed on 28, 22, and 26 ruminal solid, liquid fraction, and fecal samples from lambs, and 23, 22, and 23 samples from kids. Moringa supplementation significantly increased BWG in lambs (21.09 ± 0.78 to 26.12 ± 0.81 kg) and kids (14.60 ± 1.29 to 18.28 ± 1.09 kg) (p-value ≤ 0.01). Microbiome analysis revealed an elevated *Firmicutes:Bacteroidetes* ratio in the moringa diet group. Moringa-fed animals exhibited increased microbial genera associated with volatile fatty acids (VFAs) production (*Prevotella, Anaerovibrio, Lachnospiraceae, Butyrivibrio, Christensenella*) and starch and fiber digesters (*Proteobacteria, Ruminococcus*). The increase in the bacterial genus *Sharpea* suggested possible methane reduction and decreased proportion of pathogens, *Aliarcobacter_ID28198*, *Campylobacter_ID194* and *Campylobacter_ID1660076* suggest health benefits. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis demonstrated significant alterations in microbial gene pool and metabolic pathways related to carbohydrate, protein, lipid and energy metabolism, indicating potential improvements in animal health. Overall, moringa feeding showed higher energy recovery, improved growth, and potential benefits in methane reduction and reduced pathogenic bacteria.

Key points

- *Study assessed the effects of Moringa olifera diet on lambs and kids.*
- *Improved growth performance noted with moringa diet.*
- *Moringa feed increased Firmicutes:Bacteroidetes ratio in rumen.*

Keywords Growth performance · Moringa leaf meal · Rumen and fecal microbial diversity · Small ruminants

Introduction

Animal production systems are facing increasing demands to meet the requirements of a growing global population (Hunter et al. 2017; van Dijk et al. 2021). Feed expenses, which may account for up to 70% of total costs in the livestock industry, are pivotal in determining profitability (Kenny et al. 2018). Feed efficiency in small ruminants (sheep and goats) is an important economic trait and has a greater impact on the economic benefits of farms (Zhang et al. 2021). In addition, sustainable livestock production depends on enhancing nutrients harvested from the feed, thereby increasing the animal performance and health.

The rumen is a complex ecosystem harboring numerous microbial communities including bacteria, fungi, protozoa, archaea and bacteriophages. Based on earlier research, the ruminal microbial community was assigned to three major groups i.e. (i) microbes associated with feed particles referred to as the solid fraction, (ii) microbes associated with ruminal fluid referred to as the liquid fraction and (iii) microbes on the ruminal epithelium referred to as

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epithelium-associated microbiota (McAllister et al. 1994; Wallace et al. 1979). Microbial fermentation in the rumen plays a key role in host nutrition and affects host feed conversion efficiency. The rumen microbiome is involved in numerous biochemical functions and provides nutrients to the host including volatile fatty acids (VFAs), microbial proteins and vitamins (Elghandour et al. 2020). Propionate, butyrate and acetate are the primary VFAs produced by ruminal microbiota and altogether provide almost 70% of host energy requirements (Morgavi et al. 2015). The composition and function of rumen microbiota affects the feed efficiency in animals (Cantalapiedra-Hijar et al. 2018). Therefore, it is necessary to evaluate the effect of different diets on rumen microbiota, for sustainable and blooming animal farming. The benefits of moringa are well documented for humans and recent studies have also examined its effect on animal health and productivity (Patil et al. 2022; Su and Chen 2020).

Moringa oleifera is a native Indian subcontinent plant, can grow well on hot and dry lands and humid tropics, tolerant to a wide range of rainfall (250 mm-3000 mm) (Bhargave et al. 2015) and is also little affected by drought (Sultana 2020). Moringa leaves are known for their immense nutritional value (Anwar et al. 2007). The inclusion of *M. oleifera* seeds in a beef cattle diet reduced methane production (Lins et al. 2019). Previous reports also highlighted that using moringa as a green fodder either individually or in combination with other crops or in concentrate mix increases the productive performance (growth rate and milk yield) of ruminants and does not have any negative impact on animal health (Amad and Zentek 2023).

Several studies have described the beneficial effects of moringa feed, such as improv production and performance traits, lowering methane emissions, and enhancing digestibility in small ruminants including kids and lambs (Gebregiorgis et al. 2012; Kholif et al. 2016, 2018, 2022; Leitanthem et al. 2022a, b). But very limited research has been carried out to understand its effect on the composition and functions of ruminal and fecal microbiome (Ebeid et al. 2020; Jadhav et al. 2018)**.** By using metagenome shotgun sequencing it possible to inspect the composition of rumen microbial diversity and their gene pool in more detail and paves the way to study the correlation between diet and rumen microbial metabolism at the genomic level (Lima et al. 2015; Pitta et al. 2016). Therefore, the present study aimed to, 1) assess the effect of moringa leaf meal (MLM) on the performance (body weight) of small ruminants and2) to investigate the effect of the same on the ruminal and fecal microbiota composition and their gene pool. In this study, we fed lambs and kids with 17% of MLM to see its impact on their body weight gain and then applied shotgun metagenomics to test our hypothesis how MLM feed affects the ruminal and fecal microbial community structure and changes in functional genes. We further correlated the shift in the rumen microbiome with body weight gain in small ruminants.

Material and methods

Ethical approval

The experiment was conducted at CAZRI (Central Arid Zone Research Institute), Jodhpur, India. Animal care and welfare compliance with the experiment were performed according to institutional animal ethical guidelines.

Experimental design and sample collection

A total of 28 lambs (*Ovis aries*) and 24 kids (*Capra hircus*) were subjected to an individual stall-feeding experimental trial and randomly grouped into two groups i.e. T1- no-moringa and T2-17% moringa for both lambs and kids. In lambs, each 14 animals were assigned to T1 and T2, whereas in kids, there were 12 animals in each group. The feed composition of the no-moringa and 17% moringa groups are provided in Supplementary Table S1. The 17% moringa leaf meal addition was selected to create isonitrogenous diets for both groups.The feeding management for the T1 group involved providing a fodder mixture composed of Massor (*Lens culinaris*) straw, *Prosopis cineraria*, and Guar phalgati (*Cyamopsis tetragonoloba*) in a 50:25:25 ratio, with a nutritional content of 10% crude protein (CP) and 52.5% total digestible nutrients (TDN). The T2 group were fed a total mixed ration (TMR) with a roughage mixture (60 parts) of the same components as the T1 group and a concentrate mixture (40 parts) as detailed in Supplementary Table S1, resulting in a nutritional content of 13.57% CP and 60.57% TDN. Both T1 and T2 groups were provided at a fixed rate of 650 g per head per day (g/h/d) for the first 74 days, and then at 750 g/h/d from day 75 until the end of the experiment. The experiment was conducted from 16.01.2022 to 02.08.2022 (almost 200 days) and samples were collected at the end of the experiment. Rumen contents and fecal samples were collected by trained veterinarians. The rumen contents were obtained through a stomach tube and filtered through sterilized four layered cheese cloth to make solid and liquid fractions separately and stored in RNA protectant bacteria reagent (Qiagen, Valencia, CA, USA). Fecal samples were collected from the rectum into sterile containers filled with RNA protectant bacteria reagent (Qiagen, Valencia, CA, USA). All the samples were stored at -80 °C until DNA extraction. During the entire experiment, the body weight of the animals was measured fortnightly.

DNA extraction, library construction and metagenome sequencing

Total DNA extraction from rumen solid and liquid fractions and fecal samples was performed using a QIAmp fast DNA stool mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The rumen solid fraction and fecal samples were pretreated with sterilized phosphate buffer saline (PBS) and 0.1% (v/v) Tween 20 to dislodge the fiber and fecal particle adherent bacteria. DNA integrity was evaluated by 0.8% agarose gel and quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Shotgun metagenomics libraries were constructed using a QIAseq FX DNA library kit (Qiagen, Hilden, Germany) and sequencing was performed using paired end chemistry (250 × 2) chemistry on an Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) at the GBRC NGS facility, Gujarat, India.

Bioinformatics analysis of metagenome sequences

The (.bcl) files from NovaSeq 6000 were converted to FASTQ files using the DRAGEN server (v. 07.021.645.4.0.3) with the option (–bcl-conversion-only true) (Illumina, San Diego, CA, USA). This step removes the adapter sequences during the demultiplexing process (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Andrews 2010). The quality of the obtained raw data was assessed using the FASTQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The Illumina forward and reverse reads were merged with bbmerge.sh (https://github.com/BioInfoTools/BBMap) to generate single reads from overlapping paired end reads (Bushnell et al. 2017). From the samples having > 1 million reads, one million reads were randomly subsampled using the seqtk tool (https://github.com/lh3/seqtk). Then reads were mapped against the AnnoTree database (https://software-ab.cs.uni-tuebingen.de/download/megan-annotree/welcome.html) using DIAMOND BLASTx, with a minimum percent identity of 60% and an e-value of 1E-5. The resulting (.daa) files were processed using daa-meganizer which maps AnnoTree accessions to taxonomic and functional classes (NCBI and KEGG) (Gautam et al. 2022). These files were then imported into MEGAN6 (Community Edition, version 6.24.22 built 2023), and the phylogenetic tree was visualized (Huson et al. 2016). The results were exported as (.spf) files for further statistical analysis.

Statistical analysis

Statistical analysis of metagenomic profiles (STAMP) was used to analyze microbial communities and their functional profiles (Parks et al. 2014). We performed Welch's two-sided *t*-test between no-moringa and 17% moringa groups with 0.95% confidence interval and multiple correction test with the Benjamini–Hochberg FDR approach. Features with *p*-value- ≤ 0.05 were considered significant. Alpha diversity and beta diversity of taxonomic profiles were determined using the MOCHI online tool (Zheng et al. 2022). Alpha diversity was measured through Shannon and Simpson diversity and evenness indices and statistical significance was declared at p-value ≤ 0.05 with the Kruskal–Wallis test and Tukey test for post hoc analysis. Beta diversity was calculated using permutational multivariate ANOVA (PERMANOVA) and displayed using Principal Coordinate Analysis (PCoA) and Non-Metric Multidimensional Scaling (NMDS) with p-value ≤ 0.05. LDA effect size (LEfSe) analysis was performed using the LEfSe (v1.1.2) command line tool to identify the discriminant taxonomical and functional features between two groups, where thresholds were as follows: Linear Discriminant Analysis Effect Size (LDA) score > 3.0 and p-value ≤ 0.05. The body weight data of lambs and kids were analyzed using a linear mixed model statistical method in SPSS (version 27; SPSS Inc., Chicago, IL, USA) and the results are represented as the mean ± standard error.

Results

Growth performance between diets with and without *moringa*

The growth performance (BWG) of lambs and kids fed with and without moringa diet is shown in Table 1. Lambs (26.12 ± 0.81 kg vs 21.09 ± 0.78 kg, p-value ≤ 0.01) and kids (18.28 ± 1.09 kg vs 14.60 ± 1.29 kg, p-value ≤ 0.01) fed 17% moringa diet showed significant increase in body weight when compared with no-moringa diet. Additionally, improved average daily weight gain (ADG) was observed in both lambs [78.07 g/h/d (moringa) vs 48.23 g/h/d (no-moringa)] and kids [59.28 g/h/d (moringa) vs 30.98 g/h/d (no-moringa)], along with improved feed conversion ratios (FCR) for lambs [9.08 (moringa) vs 14.10 (no moringa)] and kids [(13.81 (moringa) vs 22.87(no moringa)] when

Table 1 Effect of 17% moringa leaf meal on body weight of lambs and kids

Animal	Group	Mean ± S.E	*F*-statistics	*p*-value
Lambs	No-moringa	21.09 ± 0.78	21.179	0.000*
	17% moringa	26.12 ± 0.81		
Kids	No-moringa	14.60 ± 1.29	9.606	0.006*
	17% moringa	18.28 ± 1.09		

compared moringa with no-moringa diet (Supplementary Table S2).

Sequencing data generated for lambs and kids

We generated a total of 181.44 GB sequencing data for this study. In the lambs and kids groups, on average > 1 million reads per sample were generated for each experiment except, in the no-moringa group fecal samples (~ 0.83 million) (Supplementary Table S3).

An outline of ruminal bacterial composition and abundance

The gradually leveled off rarefaction curves depicted in Supplementary Figure S1, indicated a sufficient amount of sequencing data for further downstream analysis. From the rumen liquor samples of lambs, we identified 280 and 267 different taxa in the solid and liquid fractions, respectively (Supplementary Table S4A and B). The kid group exhibited 648 and 597 distinct taxa in their solid and liquid fractions, respectively (Supplementary Tables S4D and E). For lambs, solid fraction samples revealed 22 and 26 distinct bacterial genera in the no-moringa and 17% moringa groups, respectively, with each genus representing an abundance of over 1% of the total sequences in at least one sample (Supplementary Table S4A). From the liquid fraction samples for lambs, we identified 21 and 26 bacterial genera in the no-moringa and 17% moringa groups, respectively with the above-mentioned threshold (Supplementary Table S4B). Similarly, in the case of kids, we exclusively detected 27 bacterial genera in the solid fraction of the no-moringa group and 26 in the 17% moringa group (Supplementary Table S4D). From the liquid fraction samples for kids, 30 genera were exclusively found in the no-moringa group, while 24 were exclusive to the 17% moringa group (Supplementary Table S4E).

The phylum *Bacteroidetes* was found to be most populous in the lamb and kid rumen after *Firmicutes*, *Verrucomicrobia*, *Proteobacter* and *Lentishaerae* (Table 2 and Supplementary Figures S2 and S3). We also identified other phyla (abundance was < 1%) such as *Fibrobacteres*, *Spirochaetes*, *Synergistetes*, *Actinobacteria* and *Euryarchaeota*. *Bacteroidetes* were found to be more abundant in lambs fed on without a moringa diet when compared with kids. However, *Firmicutes* were more abundant in both lambs and kids fed 17% moringa and interestingly, the *Firmicutes*: *Bacteroidetes* ratio was comparatively high in animals fed 17% moringa (Table 2). Similarly, the prevalence of phylum *Proteobacter* was increased in animals fed 17% moringa (Table 2). On the other hand, *Lentisphaerae*, *Verrucomicrobia* and *Euryarchaeota* were reduced in lambs but increased in kids fed 17% moringa diet.

At the genus level, on average (solid and liquid samples), the most abundant genus was *Prevotella* (Table S4), and its abundance was marginally increased in lambs (37.46%) fed 17% moringa compared with kids (22.54%) and those fed without a moringa diet (lambs- 33.73% and kids- 22.21%). Intriguingly, *Selenomonas* genera showed a downward trend in lambs (2.38%) and kids (2.08%) fed a 17% moringa diet compared with those fed a diet without moringa (lambs- 5.04% and kids- 4.20%).

Table 2 Phylum level abundance (in percentage) of the ruminal microbial community in no-moringa and 17% moringa fed group in lambs and kids

Phylum	No-moringa				17% moringa			
	Lambs-solid	**Lambs-liquid**	**Kids-solid**	**Kids-liquid**	**Lambs-solid**	**Lambs-liquid**	**Kids-solid**	**Kids-liquid**
Bacteroidetes	60.90	58.95	50.40	47.54	55.91	52.97	48.86	37.78
Firmicutes	14.66	16.60	21.87	19.16	20.62	19.97	23.52	25.48
Firmicutes: *Bacteroidetes* ratio	0.24	0.28	0.43	0.40	0.36	0.37	0.48	0.67
Verrucomicrobia	1.99	2.20	2.49	5.02	0.80	0.88	2.44	5.90
Proteobacter	1.14	1.22	1.03	1.03	4.93	9.08	2.40	2.33
Lentisphaerae	1.07	1.04	0.99	1.87	0.48	0.56	1.25	2.88
Fibrobacteres	0.63	0.19	0.64	0.34	0.67	0.27	0.68	0.43
Spirochaetes	0.57	0.33	0.64	0.57	0.49	0.29	0.53	0.19
Synergistetes	0.33	0.53	0.41	0.38	0.09	0.07	0.25	0.32
Actinobacteria	0.23	0.09	0.37	0.35	0.39	0.33	0.52	1.05
Euryarchaeota	0.12	0.15	0.48	0.29	0.11	0.08	1.04	0.45

Comparative ruminal microbiota profiles between the no-*moringa* and 17% *moringa* groups

PCoA and NMDS of the genus level classification of the microbiome profile based on the Bray–Curtis similarity distance matrix and PERMANOVA statistics, showed that the ruminal bacterial community composition differed significantly (p-value ≤ 0.01) between animals fed with and without moringa both in solid and liquid fractions of lambs (Fig. 1A, B, 2A and B) and kids (Fig. 3A, B, 4A and B). Alpha diversity indices (Simpson diversity, Shannon diversity and evenness approach) in rumen solid and liquid fractions of lambs and kids were significantly (p-value ≤ 0.05) different between diets with and without moringa (Fig. 1C-F, Fig. 2C-F, Fig. 3C-F and Fig. 4C-F).

LEfSe analysis was performed with LDA scores ≥ 3.0 to identify specific bacterial taxa that were characteristic of each feeding group (moringa and no-moringa) both in lambs and kids separately. We observed that 23 bacterial taxa differed significantly in the rumen solid fraction of lambs, 14 from the 17% moringa diet and nine from the without moringa diet (Fig. 1G and Supplementary Table S5). In the liquid fraction samples, 25 significantly different bacterial taxa, 15 in the 17% moringa diet group and 10 in the without moringa diet group, were identified (Fig. 2G and Supplementary Table S5). Bacterial taxa such as *Prevotellaceae_ID224577*, *Prevotella_ID838*, *Proteobacter_ID47936* and *ID1224*, *Succinivibrionaceae_ID83763*, *Ruminobacter_ID866*, *Eubacteriales_ID186802* and *ID50559*, *Lachnospiraceae_ID186803* and *ID186928*, *Ruminococcus_ID1263*, *Sharpea_ID519427* and *Selenomonas_ID970* dominated in the rumen solid and liquid fractions of lambs fed 17% moringa diet, while *Bacteroidetes_ID976*, *Bacteroidales_ID171549*, *ID189693* and *ID185291*, *Lentisphaerae_ID278094*, *Verrucomicrobia_ID417295* and *Selenomonadales_ID1806835* were dominant in the rumen solid and liquid fractions of lambs fed a no-moringa diet (Figs. 1H, 2H). Additionally, *Butyrivibrio_ID830* was found to be significantly differentiated in the 17% moringa diet especially in the rumen solid fraction, whereas

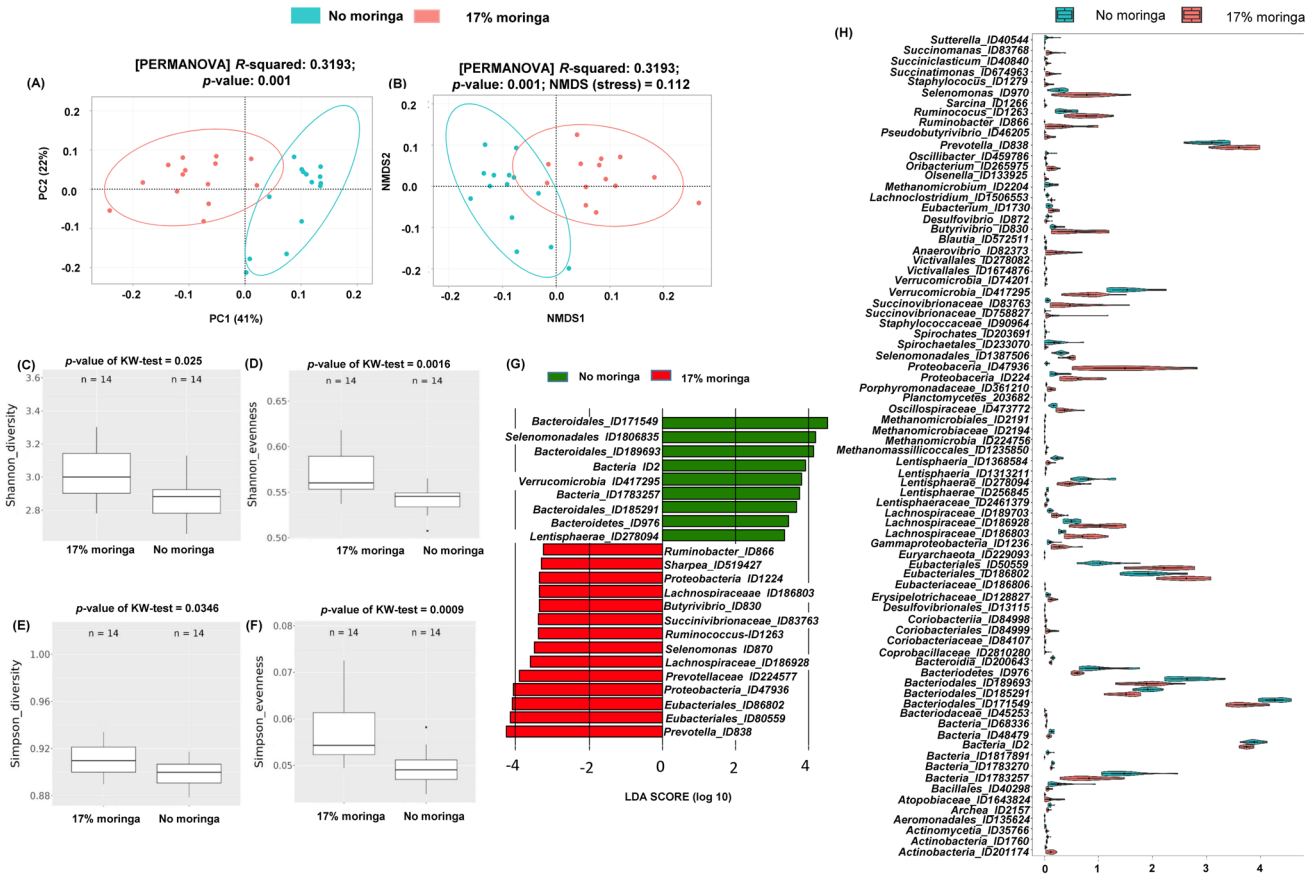

Fig. 1 Analysis of taxonomic metagenomic data of solid fraction of lambs' rumen liquor between no-moringa and 17% moringa feed groups. (**A**) PCoA plot of bacterial community. (**B**) NMDS of bacterial community. (**C**-**F**) Alpha diversity: based on Simpson and Shannon diversity and Simpson and Shannon evenness. (**G**) Linear discrimination analysis (LDA) effect size (LEfSe). (**H**) Box + violin plot of significantly differentiated abundance profiles of bacterial genera

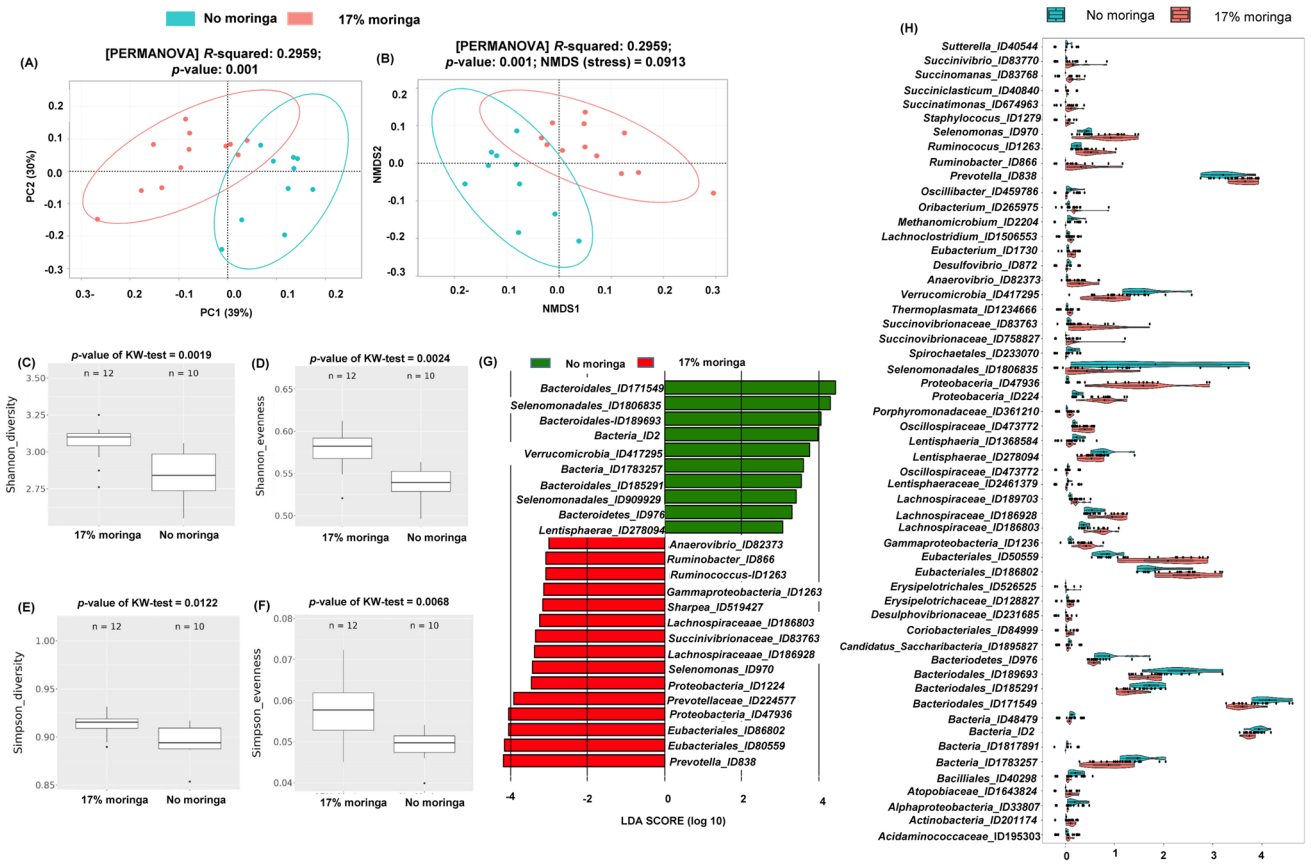

Fig.2 Analysis of taxonomic metagenomic data of liquid fraction of lambs' rumen liquor between no-moringa and 17% moringa feed groups. (**A**) PCoA plot of bacterial community. (**B**) Non-metric dimensional scaling (NMDS) of bacterial community. (**C**-**F**) Alpha diversity: based on Simpson and Shannon diversity and Simpson and Shannon evenness. (**G**) Linear discrimination analysis (LDA) effect size (LEfSe). (**H**) Box + violin plot of abundance profiles of bacterial genera

Gammaproteobacteria_ID1236 and *Anaerovibrio_ID82373* were in the rumen liquid fraction.

A total of 15 bacterial taxa differed significantly in the rumen solid fraction of kids, eight from the 17% moringa diet and seven from the without moringa diet (Fig. 3G and Supplementary Table S6). A total of 21 differentially abundant genera were observed in the rumen liquid fraction samples, 11 in the 17% moringa diet group and 10 in the no-moringa diet group (Fig. 4G and Supplementary Table S6). *Uncultured Prevotellaceae bacterium_ID370804*, *Prevotellaceae bacterium UBA2738_ID1952469*, *Bacteroidales bacterium UBA3289_ID1950477*, *uncultured Eubacteriales bacterium_ID172733* and *Lachnospiraceae_ID186803* were significantly dominant in the 17% moringa diet kids in both rumen solid and liquid fraction samples (Figs. 3H, 4H). *Uncultured Proteobacterium_ID153809*, *Succinivibrionaceae_ID83763*, *Lachnospiraceae_ID186803* and *Methanobrevibacter_ID2172* were specifically abundant in the rumen solid fraction, while *Desulfovibrionaceae bacterium UBA3823_ID1961562*, *uncultured Lentisphaerae bacterium_ID278095*, *Actinobacteria_ID201174*, *Christensenella_ID1946267* and *uncultured Selenomonadales bacterium_ID1387507* were dominant in the rumen liquid fraction of the 17% moringa diet kids. In the no-moringa diet group, *Bacteroidetes_ID976*, *Bacteroidales_ID171549*, *Bacteroidales_ID185291*, *Bacteroidales bacterium UBA3868_ID1950533*, *Selenomonadales_ID 909929* and *Selenomonadales bacterium UBA7018_ID1951243* were significantly dominant in kids fed the no-moringa diet in both rumen solid and liquid fractions. In addition, *Prevotellaceae_ID171552*, *Prevotella_ID838* and *Prevotellaceae bacteria UBA7017_ID1952525* were found to be dominant in the rumen liquid fraction of the no-moringa diet group. In the comparison of lambs and goats in the 17% moringa feed group, *Succinivibrionaceae_ID83763* and *Lachnospiraceae_ID186803* were common in the rumen solid fraction. However, *Eubacteriales_ID186802* and *Lachnospiraceae_ID186803* were common in the rumen liquid fraction. Similarly, in the no-moringa feed group, *Bacteria_ID2*, *Bacteroidetes_ID976*, *Bacteroidales_ID171549* and *Bacteroidales_ID185291* were

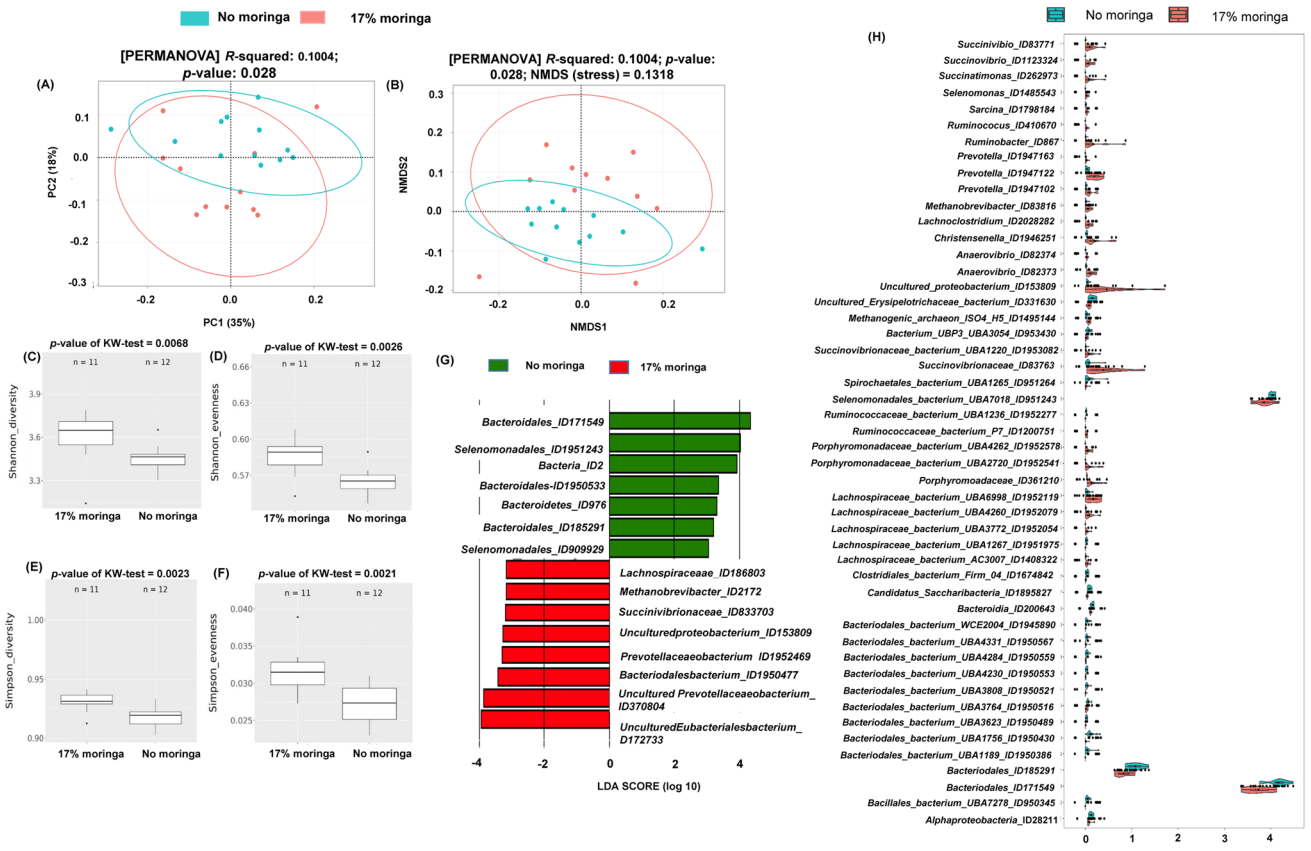

Fig.3 Analysis of taxonomic metagenomic data of solid fraction samples of kids' rumen liquor between no-moringa and 17% moringa feed groups. (**A**) PCoA plot of bacterial community. (**B**) NMDS of bacterial community. (**C**-**F**) Alpha diversity: based on Simpson and Shannon diversity and Simpson and Shannon evenness. (**G**) Linear discrimination analysis (LDA) effect size (LEfSe). (**H**) Box + violin plot of abundance profiles of bacterial genera

common in the rumen solid and liquid fractions, while *Selenomonadales_ID909929* was common in the rumen liquid fraction.

Functional potential of bacterial communities between both diets

The KEGG pathways of ruminal bacterial communities were used to compare the functional potential of the microbiome with and without moringa diet fed lambs and kids (Supplementary Tables S7 A, B, D and E). In lambs, genes involved in pathways related to K1000500: Starch and sucrose metabolism, K1002010: ABC transporters, K1002020: Two-component system, K1000190: Oxidative phosphorylation, K1000564: Glycerophospholipid metabolism, K1000760: Nicotinate and nicotinamide metabolism, K1000623: Toluene degradation, and K1000480: Glutathione metabolism were significantly enriched in rumen solid fraction of 17% moringa group (Supplementary Figure S4, Supplementary Table S7A). On the other hand, genes associated with pathways related to K1000051: Fructose and mannose metabolism, K1000280: Valine, leucine and isoleucine degradation, K1000540: Lipopolysaccharide biosynthesis, K1000030: Pentose phosphate pathway, K1000061: Fatty acid biosynthesis, K1000071: Fatty acid degradation, and K1000410: beta-alanine metabolism were more abundant in rumen solid fraction of lambs fed on without moringa diet. In the rumen liquid fraction, K1000190: Oxidative phosphorylation, K1000480: Glutathione metabolism, K1000622: Xylene degradation and K1000564: Glycerophospholipid metabolism were more abundant in the 17% moringa diet, while K1000970: Aminoacyl-tRNA biosynthesis, K1000051: Fructose and mannose metabolism, K1000520: Amino sugar and nucleotide sugar metabolism, K1000540: Lipopolysaccharide biosynthesis, K1000010: Glycolysis/ Gluconeogenesis and K1000030: Pentose phosphate pathway, were significantly abundant in the no-moringa diet (Supplementary Figure S5, Supplementary Table S7B).

The genes involved in KEGG pathways associated with K1000520: Amino sugar and nucleotide sugar metabolism, K1000040: Pentose and glucuronate interconversions, K1000010: Glycolysis/ Gluconeogenesis and K1000640:

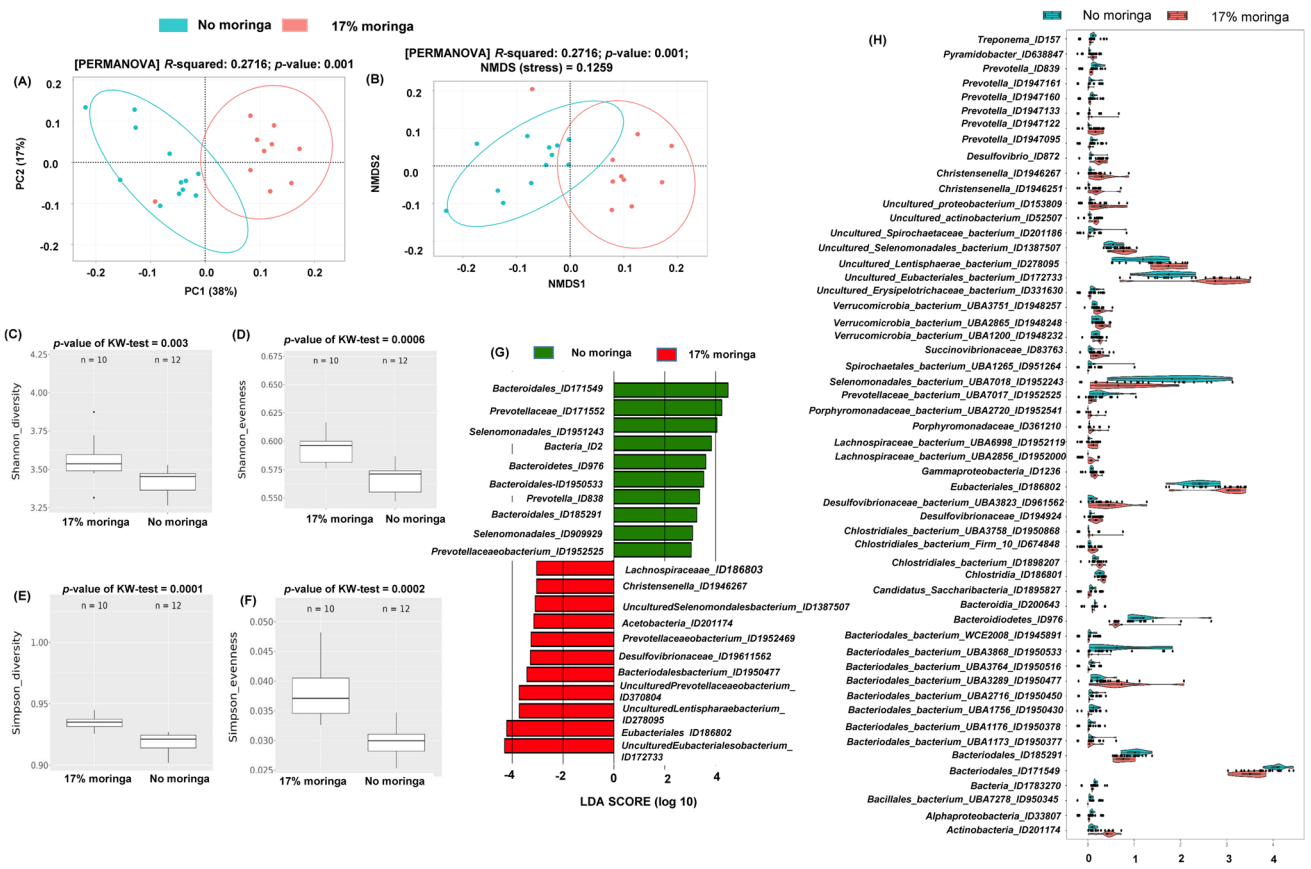

Fig.4 Analysis of taxonomic metagenomic data of liquid fraction samples of kids' rumen liquor between no-moringa and 17% moringa feed groups. (**A**) PCoA plot of bacterial community. (**B**) Non-metric dimensional scaling (NMDS) of bacterial community. (**C**-**F**) Alpha diversity: based on Simpson and Shannon diversity and Simpson and Shannon evenness. (**G**) Linear discrimination analysis (LDA) effect size (LEfSe). (**H**) Box + violin plot of abundance profiles of bacterial genera

Propanoate metabolism were significantly enriched in the rumen solid fraction of kids fed without moringa diet (Supplementary Figure S6, Supplementary Table S7D). In the rumen liquid fraction of kids, K1000561: Glycerolipid metabolism, K1000760: Nicotinate and nicotinamide metabolism, K1000630: Glyoxylate and dicarboxylate metabolism, K1000250: Alanine, aspartate and glutamate metabolism, K1000564: Glycerophospholipid metabolism, K1000020: Citrate cycle (TCA cycle), K1000650: Butanoate metabolism, K1000260: Glycine, serine and threonine metabolism, K1000030: Pentose phosphate pathway, K1001051: Biosynthesis of ansamycins, K1000052: Galactose metabolism, and K1000910: Nitrogen metabolism were enriched more in the 17% moringa diet (Supplementary Figure S7, Supplementary Table S7E).

Dynamics of fecal microbiota composition between the two diets

Rarefaction curves (Supplementary Figure S8) determined an even distribution of fecal microbial species and sufficient amount of sequencing data for further downstream analysis. *Firmicutes* (37.86%) and *Proteobacter* (10.44%) were found to be more abundant and dominate the fecal microbiota composition in lambs fed the moringa diet than in those fed the no-moringa diet (*Firmicutes*-35.62% and *Proteobacter*-9.77%), while *Bacteroidetes* dominated in the no-moringa diet group (19.70%) as compared to the 17% moringa group (16.86%) (Table 3 and Supplementary Figure S9A). Accordingly, in kids, *Firmicutes* (36.67%) and *Bacteroidetes* (15.81%) were predominant in the no-moringa group, while *Proteobacteria* (17.16%), *Verrucomicrobia* (6.53%) and *Actinobacteria* (1.64%) were predominant in the 17% moringa diet group (Table 3 and Supplementary Figure S9B).

The main bacterial genera in the fecal samples of lambs and kids fed both the diets were *Bacteria-ID2*, *Eubacteriales-ID186802*, *Firmicutes-ID1239*, *Bacteroidales-ID171549*, *Enterobacteriaceae_ID543*, *Eubacteriales_ID50559* and *ID39779*, *Alistipes-ID239759* and *Akkermansiaceae-ID1951308* and *ID1647988* (Supplementary Table S4C, F). Alpha diversity indices (observed, Shannon and Simpson evenness approach) were compared

Table 3 Abundance of microbial phyla (in percentage) in fecal samples of lambs and kids in no-moringa and 17% moringa feed groups

Phylum	No-moringa		17% moringa	
	Lambs-fecal	**Kids-fecal**	**Lambs-fecal**	**Kids-fecal**
Firmicutes	35.62	36.67	37.86	32.73
Bacteroidetes	19.70	15.81	16.86	14.19
Proteobacteria	9.77	10.74	10.44	17.16
Verrucomicrobia	3.09	4.08	3.06	6.52
Actinobacteria	1.07	1.16	0.71	1.64
Euryarchaeota	0.96	0.66	0.87	0.29
Lentisphaerae	0.59	0.43	0.62	0.31
Spirochaetes	0.46	0.343	0.32	0.21

between with and without moringa diet fed lambs and kids. In fact, significant differences for all alpha diversity measures (p-value ≤ 0.05) except for Simpson evenness were observed between lambs fed diets with and without moringa (Supplementary Figures S10C-F). No significant differences in alpha diversity measures were observed between kids fed no-moringa and 17% moringa diet (Supplementary Figures S11C-F). Similarly, beta diversity analysis based on Bray–Curtis distance showed no significant difference between with and without moringa diet fed lambs and kids (Supplementary Figures S10A-B and S11A-B).

At the genus level, in lambs a total of 11 taxa were found to have significant differences through LEfSe analysis (LDA score ≥ 3.0 and p-value ≤ 0.05) (Supplementary Figure S10G and Supplementary Table S8). *Desulfovibrionaceae_ID194924*, *Firmicutes_ID1239*, *Eubacteriales_ID186802*, *ID50559* and *ID39779* genera were dominant genera and were significantly more abundant in lambs fed 17% moringa diet, whereas *Aliarcobacter_ID28198*, *Campylobacter_ID194*, *Campylobacter_ID1660076* and *Mycolicibacterium_ID1566886* were significantly more abundant in lambs fed the no-moringa diet (Supplementary Figure S10H). No significant discriminant genera were obtained in kids fed diets with and without moringa.

Functional analysis of fecal microbiota

To compare the functional genes of fecal microbiota between lambs and kids fed with and without moringa diet, we first tested the KEGG pathway enrichment analysis based on the top-abundance KOs (mean relative abundance > 0.01%). The significantly enriched KEGG metabolism pathways in each group are shown in Supplementary Figures S12 and S13, and Supplementary Tables S7C, S7F. Due to differences in the diet, the metabolic pathway enrichment was different in the two feed groups. K1000250: Alanine, aspartate and glutamate metabolism, K1000910: Nitrogen metabolism, K1001051: Biosynthesis of ansamycins, K1000500: Starch and sucrose metabolism and K1000052: Galactose metabolism, were enriched in lambs fed 17% moringa diet, while K1004020: Calcium signaling pathway, K1005340: Primary immunodeficiency and K1005012: Parkinson's disease were enriched in the no-moringa diet fed lambs (Supplementary Figures S12, Supplementary Table S7C). In addition, K1000910: Nitrogen metabolism and K1000780: Biotin metabolism were relatively more enriched in kids fed 17% moringa diet, whereas K1000680: Methane metabolism, K1000620: Pyruvate metabolism, K1000010: Glycolysis/Gluconeogenesis, K1000970: Aminoacyl-tRNA biosynthesis and K1000710: Carbon fixation in photosynthetic organisms were enriched in kids (Supplementary Figure S13 and Supplementary Table S7F) that do not have moringa in their diet.

Discussion

The interaction between diet and the host microbiome has been shown to be associated with animal health and performance. Thus, the usage of various plant biomasses with beneficial effects has been reported to regulate the microbiota composition to improve animal performance (Liu et al. 2021). Previous studies have shown the beneficial effects of feeding moringa in livestock farming (Silva et al. 2021). Nevertheless, minimal light has been shed on its impact on animal's gut microbiota composition. By considering the above facts, we aimed to determine the response of rumen and fecal microbial communities toward moringa supplementation using metagenome analysis in lambs and kids and their growth performance.

The results of the present study demonstrated that feeding moringa to lambs and kids enhanced growth performance in terms of body weight gain. In addition, improved ADG, FCR and per kg weight gained with TDN intake as well as total CP intake was also observed. These results are consistent with previous studies (Babiker et al. 2017; Leitanthem et al. 2022b; Wankhede et al. 2022). In this study, *Bacteroidetes*, *Firmicutes* and *Proteobacter* were the dominant phyla in both lambs and kids irrespective of the diet. These findings are corroborated by earlier reports on ruminal microbial communities in ruminants (Morgavi et al. 2015; Wang et al. 2017; Zhang et al. 2021). *Bacteroidetes* and *Firmicutes* are the two important core components of the rumen microbiome encoding enzymes that promote the breakdown of complex polysaccharides followed by the synthesis of VFAs (Jami et al. 2013). The trend of increased *Firmicutes*:*Bacteroidetes* ratio and body weight

was observed in the 17% moringa group in kids and lambs when compared with those fed without a moringa diet. The increased ratio has been associated with reduced residual feed intake and improved host energy absorption and storage from the diet (Wang et al. 2017; Zhang et al. 2021). In this study also, we have noted improved FCR in the animals fed with moringa based diet. The improved body weight in animals fed 17% moringa diet could be the reason for the increase in *Firmicutes* abundance, which further increases short-chain fatty acid (SCHA) production (Wei et al. 2022). These factors have been previously associated with body weight in goats (Min et al. 2019). Therefore, we hypothesized that an increased *Firmicutes:Bacteroidetes* ratio could be the reason for improved body weight in animals fed 17% moringa diet. A positive correlation between *Proteobacteria* and starch concentration in the diet has already been established (Dong et al. 2021), and the increased abundance of *Proteobacteria* in the 17% moringa diet reflected the increased starch digestion.

Prevotella is one of the core genera involved in the breakdown of lignocelluloses and other plant fibers in the goat rumen (Dao et al. 2021; Li et al. 2020; Tian et al. 2022; Zhang et al. 2021). Members of genus the *Prevotella* play pivotal roles in the digestion of cellulose, hemicellulose, pectin and starch (Dao et al. 2021) and important bacterial genera involve in breakdown of oligopeptides into amino acids, a rate limiting step in rumen proteolysis (Calsamiglia et al. 2010). Members of this genus have been reported to decrease the accumulation of lactate and increase the production of succinate and propionate (Rodríguez 2003). Furthermore, propionate is the main precursor of gluconeogenesis, which contributes 60–80% of the net glucose supply in dairy cows (Reynolds et al. 2003). In this study, based on LEfSe analysis, we found that *Prevotella* serves as a keystone taxon of microbial species in the rumen microbiome of 17% moringa fed lambs and kids groups. Together, this data suggests that the rumen microbiome of lambs and kids fed 17% moringa has a higher feed conversion efficiency by degrading complex plant polysaccharides to meet the energy requirements of the host. However, in contrast to these findings, some studies also identified various *Prevotella* species associated with low average daily gain and other growth performance traits, which had a negative effect on rumen fermentation efficiency in dairy goats (Wang et al. 2023). The functional diversity within the *Prevotella* genus and their interaction with other microbial species in the rumen (Hitch et al. 2022) could be the reason for these conflicting results.

The higher abundance of *Succinivibrionaceae* and *Anaerovibrio,* which are crucial for propionate production, in lambs and kids fed a 17% moringa diet suggests that these microbes could play a significant role in providing energy to the host. The members of the *Succinivibrionaceae* family commonly produce succinate, which is the precursor of propionate, and *Anaerovibrio* has been reported to produce propionate through lipid metabolism (Lv et al. 2020; Prive et al. 2013). Additionally, *Lachnospiraceae* and, *Butyrivibrio*, which are enriched in lambs' and kids' fed 17% moringa diet are known for butyrate production in the rumen (Haas and Blanchard 2017; Kopecny et al. 2003) which can promote the growth of rumen papillae (Gorka et al. 2009). The higher abundance of *Christensenella_ID1946267* in the liquid fraction of kids fed 17% moringa diet has also been associated with fermentation of structural carbohydrates to produce butyric acid as a main end product (Xiong et al. 2019). The genus *Ruminococcus*-ID1263 was enriched in both solid and liquid fractions of 17% moringa group lambs. *Ruminococcus*, *Butyrivibrio*, *Prevotella*, and *Succinivibrio* are linked to feed efficiency traits, including average daily gain (ADG) and feed conversion ratio (FCR), in heifers and beef cattle (Carberry et al. 2012; Lu et al. 2023; Myer et al. 2015; Na and Guan 2022). Overall, these results suggest, feeding moringa to animals leads to increased energy harvest from the feed and improved body weight gain. This process might involve the enrichment of plant fiber degraders as well as volatile fatty acids (VFA) producers and associated bacteria.

Interestingly, we noticed a relatively higher abundance of the genus *Sharpea-ID519427* in the solid and liquid fractions of lambs fed 17% moringa diet. Previously, it has been reported that the increase proportion of lactate producing *Sharpea* in sheep rumen can lead to lower methane emissions due to changes in fermentation patterns (Kamke et al. 2016). Therefore, we hypothesized that moringa in the diet could shape the bacterial community which mitigates methane emission. However, an additional study is needed to confirm this, as in this study we have not measured the methane production under two dietary regimes. Dietary changes also modified the relative abundance of bacterial communities in the distal gut. In the present study, we observed a relatively high abundance of *Campylobacter_ID194* and *Campylobacter_ID1660076*, genera belonging to the *Campylobactereaceae* family, and of *Aliarcobacter_ID28198* in the fecal samples of lambs and kids fed without moringa diet when compared with those fed the 17% moringa diet, all these are potential zoonotic pathogens (Çelik et al. 2022; Suman Kumar et al. 2023) which can spread through feces and endanger the environment (Ogden et al. 2009). On the other hand, the abundance of these microbes was lower in animals fed by 17% moringa. Hence, moringa could reduce the abundance of pathogenic bacteria in the large intestine of the small ruminants.

Changes in the relative abundance of ruminal microbial communities also reflected the significant enrichment of different KEGG pathways and functional genes in two different diets. In the present study, we observed that the microbial communities of the moringa-based diet showed

enrichment of carbohydrate metabolism (Starch and sucrose metabolism) and ABC transporters in the rumen solid fraction. Additionally, lipid metabolism (Glycerophospholipid metabolism) was enriched in both the solid and liquid fractions of the moringa group when compared to the without moringa group. Moringa leaves could be considered a high digestible plant material that increases the abundance of bacteria involved in carbohydrate metabolism (Wang et al. 2020). Digested carbohydrates are taken up by bacterial cells majorly via ABC transporters (Bond et al. 2012; Higgins 2001) for subsequent intracellular digestion, contributing to enhanced rumen digestibility upon release of the digested products. Furthermore, glycerophospholipid metabolism has been reported to be involved in the production of VFA (Nafikov and Beitz 2007). Meanwhile, an enhanced genepool associated with carbohydrate and energy metabolism [Galactose metabolism, Citrate cycle (TCA cycle)], and lipid metabolism (Glycerophospholipid metabolism) in liquid fraction of kids fed on moringa diet demonstrated that the 17% moringa diet might enrich the gene pool involved in different metabolism pathways to meet the energy requirements of the animal. Additionally, protein metabolism (Glycine, serine and threonine metabolism and Nitrogen metabolism) and vitamin metabolism (Nicotinate and nicotinamide metabolism) pathways were also enriched in the rumen liquid of the 17% moringa-fed group. This shift is likely due to the high protein and vitamin content (Abbas et al. 2018) in the moringa diet.

The pentose phosphate pathway was enriched more in the rumen solid and liquid fraction of lambs fed without moringa; however, it was more abundant in the 17% moringa group rumen liquid samples of kids. The outcome of the same feed on different hosts could be different as host genetics can also play a role in shaping the microbial community (Tabrett and Horton 2020). The same results we have also observed in our previous studies carried out on different breeds of cattle and buffalo (Hinsu et al. 2017; Pandit et al. 2018; Pandya et al. 2010; Pitta et al. 2014). Therefore, we suspected that lambs and kids responded differently to the same diet to meet their energy requirements.

Microbial synthesis of secondary metabolites can mediate important interactions between microbes and hosts to improve overall animal health and performance (Shah et al. 2019). The enrichment of the ansamycins category in lambs and kids fed 17% moringa diet is an example of providing a secondary metabolite with potential antimicrobial (Wrona et al. 2008), anti-tumor (Brandt and Blagg 2011) and antiviral (Song et al. 2015) activities. In addition to the host beneficial effects toward growth performance, we also observed significant enrichment of toluene and xylene degradation KEGG terms in the 17% moringa feed group. Toluene is an important organopollutant and is classified as an environmental priority pollutant by the US Environmental Protection Agency (USEPA). From our findings, the moringa diet increased the abundance of microorganisms which could degrade/utilized toluene and xylene. This can be possibly explored further for bioremediation purposes. However, altogether a different experimentation setup is required.

In summary, the results of this study revealed the potential benefits of including *M. oleifera* in the diet of small ruminants. This study shows that moringa consumption positively impacts microbial communities and metabolic pathways within the rumen and fecal samples of small ruminants, resulting in improved growth performance (body weight in this study). Additionally, moringa appears to reduce the abundance of pathogenic bacteria such as *Campylobacter* and *Aliarcobacter*, suggesting potential benefits for the environment and animal health. The study also highlighted that moringa feed may help in reducing methane emissions, possibly through enriching a bacterial genus such as *Sharpea*. However, further research is needed to validate these results and explore the benefits that moringa may bring to livestock nutrition and management.

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Availability of data and materials Raw metagenome shotgun sequence data is submitted to NCBI Short Read Archive (SRA) under BioProject number PRJNA1026504 and sample-wise SRA accession numbers are given in Supplementary Table S9. The datasets supporting the conclusions of this article are included within the article as Tables and Figures.

Declarations

Ethics approval and consent to participate Animal care and welfare compliance with the experiment were performed according to institutional animal ethical guidelines.

Competing interests Authors declare no competing interests.

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