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Altering the gut microbiome of meat goats: Feeding varying levels of hempseed meal on animal performance, rumen microbiome abundance, and methanogen community changes of meat goats

Frank Abrahamsen[1]; Byeng-Ryel Min[1]; Chuck Okere[1]; Reshma Gurung[2]; Nar Gurung[1]; Woubit Abebe[2]

[1]Department of Agricultural and Environmental Sciences, Tuskegee University, USA.

[2]Department of Pathobiology, College of Veterinary Medicene, USA.

***Corresponding Author: Frank Abrahamsen**

Department of Agricultural and Environmental Sciences, Tuskegee University, Alabama, USA.

Email: fabrahamsen@tuskegee.edu

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Keywords:
Meat goats;
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Dry matter intake;
Average daily gain;
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Methanogens.

Abstract

This study aimed to determine whether increasing the levels of Hempseed Meal (HSM) **S**upplementation would affect Dry Matter Intake (**DMI**), Average Daily Gain (ADG), and rumen microbial community diversity of growing meat goats over a 60-d feeding trial. Forty, 5-month-old castrated Boer-cross goats with an average body weight (BW) of 25.65 ± 0.33 kg were randomly assigned to one of the four treatments (n=10/treatment): control, 10%, 20%, and 30% HSM of the total diets. The forage to concentrate ratio was adjusted to 50:50. Diets were pelleted as total mixed rations. The DMI was lower for 20 and 30% HSM supplementation (P>0.05) than for the control and 10% HSM diet. While, ADG (g/d) and live weight changes (kg/d) decreased (P<0.03) with increasing inclusion rate of HSM. Based on the bacterial clustering diversity, both the control and 10% treatment groups did display a significantly less (P<0.04) diverse environment when compared to the 20% and 30% groups. The fungi diversity detected within the control treatment group is significantly less (P<0.05) than that detected within the 10% and 20% groups. The relative abundance of Bacteroidetes (47.7%), Firmicutes (32.4%), and Proteobacteria (14.9%) were the most abundant bacterial phylum. In the present study, the relative abundance of Bacteroidetes phylum was lower (P<0.01) in animals fed 10 and 30% HSM diets. In comparison, the abundance of Firmicutes phylum (P<0.05) and *Methanobrevibacter* species were greater (P<0.01) in the rumen of goats fed 20 and 30% HSM than those consuming the control and 10% HSM diets. These findings provide the optimal level of HSM supplementation (<20% as-fed). Furthermore, more work needs to be completed to determine the best feeding strategy to improve the DMI, rumen fermentation, and animal performance.

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Introduction

Hemp (*Cannabis sativa* L.) is an annual herbaceous plant found worldwide and cultivated for centuries as a fiber source [7,9,63]. Recently, hemp has become a topic of discussion as it is a plant with an extensive diversity of applications (textile, pharmaceuticals, construction, etc.), including its use in animal feed. Globally, the Food and Agriculture Organization (FAO) estimates that 32 square kilometers (km^2) of hemp are harvested, including 143 Metric Tons (MT) of hempseed, 50 MT seed oil, 93 MT cake, and 331 MT leaves are produced mainly from France, China, and Chile [24]. However, the primary producers in the world are Canada and the USA, with an estimated 315,000 and 1,160 ha, respectively, asreported by [61]. The Whole Hempseed (WHS) can be used as an animal supplement or after the treatments to remove lipid components to obtain hempseed cake (HSC) or Hempseed Meal (HSM) which could potentially be used in animal feed [3]. In general, hempseed contains over 30% oil (>80% in polyunsaturated fatty acids) and about 25% crude protein (CP), with considerable amounts of dietary fiber, vitamins, and minerals [7]. A similar value of crude protein (25% on DM basis) for hempseed was reported by European Food Safety Authority [20]. However, the authorized hemp varieties are registered in the EU's Common Catalogue of Agricultural Plant Species and have content in psychotropic *delta*9-Tetrahydrocannabinol (THC) less than 0.2–0.3% (DM basis), which is the primary psychoactive substance [29]. Additionally, hempseed contains anti-nutritional compounds (e.g., phytate) that reduce the absorption of protein and micronutrients [58,59]. However, Reggiani and Russo (2016) observed that replacing 6.4% (on DM basis) of corn and soybean with hempseed or flax seed while maintaining isonitrogenous status can increase iron availability in Alpine lactating goats. Essential oil extracts from the whole hemp plant material exhibit antimicrobial activity in most bacterial habitats from human, animal, and food sources and are even active against fungi [1,49]. However, the associative effects of HSM inclusion in the diets on dry matter (DM) intake (DMI), animal performance, and rumen micro biome diversity changes in growing meat goats are not apparent.

Hempseed cake has a low DMI, rumen DM digestibility, and total volatile fatty acids (VFA) production when compared to canola meal and soybean meal in steers and sheep [61]. Conversely, feeding WHS or HSC supplementation did not affect the final live weight or average daily gain (ADG) in steers [27], and growing cattle [30], and dairy cows [36]. Unfortunately, few publications are available on the use of HSM in meat goat diets. Still, some authors reported a positive effect on the fatty acids profile of dairy cattle (e.g., milk and cheese) with an increase of n-3 fatty acids and conjugated linoleic acid [3]. Therefore, in dairy cattle, hempseed and its by-products (oil, cake, and meal) can be used as a supplement in feed, mainly as sources of essential fatty acids and amino acids [39].

The modification in the rumen microbiota diversity due to an alteration in the diet requires a lot of attention, as it increases the metabolizable energy and microbial protein supply within the rumen, improving feed efficiency and ADG [4,5,74]. The great abundance of Firmicutes within the rumen suggests that these shifts may affect feed efficiency and improve ADG [44,45,67]. Grazing steers fed with vegetative stages of fresh wheat showed an abundance of Firmicutes in their rumen [57] compared to those provided with reproductive stages of the plant. High-quality forage diets or high levels of forage ratio in the diets commonly improved the comparative richness of Firmicutes relative to Bacteroidetes [26,57,69]. However, these results did not always expose similar fashions of rumen bacterial phylum of feedlot steers [8] and dairy cattle [32,74] compared to forage-based diets in meat goats [42,43] and non-lactating cows [13]. In the past, authors have described a change in the feed will result in changes in the profile ofthe rumen microbial community of cattle, sheep, and goats [4,15]. The primary objective of the current study was to define whether increasing levels of HSM supplementation would affect DMI, animal performance, and rumen microbial diversity in growing meat goats over a 60-day feeding trial.

Materials and methods

Animal care and use

The Institutional Animal Care and Use Committee of Tuskegee University approved all animal care and experimental procedures performed in this study (# R07-2019-5). Goats were purchased from a vendor in Texas. Upon arrival, goats were dewormed with Cydectin (moxidectin; 1 mg/kg BW; Fort Dodge Animal Health, Fort Dodge, IA, USA) and vaccinated with *Clostridium Perfringens* type C and D-Tetani Bacterin-Toxoid (Bayer Corp., Shawnee Mission, KS, USA). Goats were quarantined for 30-d and were adapted to the control total mixed ration (TMR) diets. Upon completing the quarantine period, goats were individually housed indoors in approximately 1.2 m^2 pens with plastic-coated expanded metal floors. An adjustment period of 2 weeks allowed goats to become acclimated to pen conditions and feeding routine and allowed time for proper diet adjustment before the start of the study.

Animals and diets

Thirty six, castrated Boer-cross goats, approximately 5 months of age with an average BW of 25.63 ± 0.33 kg were randomly assigned to one of the four experimental treatments (n=9/treatment) which contained different levels of HSM or grain mixes containing cracked corn and soybean meal (CCSBM): 0% HSM and 30% CCSBM; 10% HSM and 20% CCSBM; 20% HSM and 10% CCSBM; or 30% HSM and 0% CCSBM diets, where HSM replaced CCSBM pre-mixed diets, as-fed (Table 1). Grain mixes comprised cracked corn, soybean meal, HSM, molasses, and a 16:8 mineral supplement (Meat Maker, Ridley USA Inc., Montgomery, AL). Grain mixes containing HSM or CCSBM diets were commercially pelleted at the local feed mill (Electic Feed Mill, Chana Creek Rd., Electic, AL). They were offered daily at 50% of the total ration, with the remaining 50% consisting of timothy grass (*Phleum Pretense*) hay as the forage source. The hemp seed meal used in the experiment was prepared using the cold press method to extract hemp seed oil. All treatment diets were formulated to be iso-nitrogenous and meet the NRC requirements for growing meat goats [50].

Grain TMR diet and long timothy hay were offered separately by weight basis, and refusals (around >5%) were recorded daily to measure Dry Matter Intake (DMI). Used as an estimate of *ad libitum* consumption by each animal, this intake level was maintained during the adaptation period. Each diet was pelleted as a complete pellet to minimize differences in physical forms and appearances and prevent sorting by goats. Goats were given *ad libitum* access to feed and water daily. All animals were adjusted to the control TMR 3 weeks before starting the feeding period. Animals were fed twice daily at 6:00 AM and 6:00 PM. Animal Body Weight (BW) was collected before feeding on days 0, 20, 30, and 60 to calculate total BW gain and ADG.

Sample collection and laboratory analyses

Rumen fluid was collected from the goats on day 60 of the feeding period via a soft stomach tube utilizing the Drench-Mate Calf RFE (Drench-Mate, Sumas, Washington). Upon collection, samples were immediately stored at -80°C. Before analysis, samples were thawed on ice, equally pooled (weight basis) to make one of the three samples per treatment (n = 3) from the nine animals per treatment, strained through four layers of cheesecloth, and used for microbiome analysis.

Feed samples for each diet and Timothy grass hay were collected. For ease of handling and processing, composite samples were dried for 48h at 60°C in a convection oven and ground in a Thomas-Willey mill (model 4, Thomas Scientific, Philadelphia, PA) to pass through a 1-mm mesh screen. Ground composite samples were analyzed for DM, crude protein (CP), acid detergent fiber (ADF), hydrolysis fat, and total digestible nutrient (TDN) was completed according to the methods described by the American Organization of Analytical Chemists (AOAC, 1990). According to the manufacturer's recommendations, the neutral detergent fiber (NDF) was determined utilizing the ANKOM 2000 fiber analyzer (Ankom Technology, Macedonia, NY). Lignin concentration was determined according to the USDA (1970) Forage Fiber Analysis Handbook Procedure 397.

DNA extraction and 16S/ITS amplicon metagenomic sequencing

One mL aliquots of samples were centrifuged at 17,000 x *g* for 1 min. The DNA extractions were performed on approximately 100 mg of the collected pellets using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA), following the protocol described manufacturer. DNA was quantified using a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). This purified DNA sample was investigated for bacteria and fungi diversity using a metagenomic sequencing PCR method. The samples were then transported to the Research and Testing Laboratory (Mr. DNA, Shallow, TX) for PCR optimization and met genomic sequencing analyses.

For bacterial sequencing, the 16S rRNA primer pair, 515F GTGCCAGCMGCCGCGGTAA/806R GGACTACVSGGGTATCTAAT, was utilized to evaluate the microbial ecology of each sample on the Ion S5 with methods via the bTEFAP® DNA analysis service [16]. Each sample underwent a single-step 30 cycles of PCR using HotStar Taq Plus Master Mix Kit (Qiagen, Valencia, CA) were utilized under the following conditions: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute; after which a final elongation step at 72°C for 10 minutes was performed. Following PCR, all amplicon products from the same treatment were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Then, samples were sequenced utilizing the Ion S5 chemistry following the manufacturer's protocols.

For fungi sequencing, the ITS primer pair, ITS1-1F CTTGGTCATTTAGAGGAAGTAA/ ITS2RGCTGCGTTCTTCATCGATGC, was utilized to evaluate the microbial ecology of each sample on the Ion S5 with methods via the bTEFAP® DNA analysis service [8,16] (Williams et al., 2010). Each sample underwent a single-step 30 cycle PCR using HotStar Taq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the conditions of bacterial sequencing. Sequences were depleted of barcodes and primers sequences, and any sequence of <200 bp was removed. In addition, sequences with ambiguous base call and homopolymer run exceeding 6 bp were removed. Sequences were then denoised and chimeras removed. Operational taxonomic units were defined after removing singleton sequences, clustering at 3% divergence (97 % similarity) [16,22,66]. OTUs were then taxonomically classified using BLASTn against a curated NCBI database and compiled into "counts" and "percentage" files into each taxonomic level. Count files contain the actual number of sequences, while the percent files contain the relative (proportion) percentage of sequences within each sample that map to the designated taxonomic classification.

Statistical analysis

For experimental treatment, linear dose levels of HSM treatments were analyzed using polynomial regression (linear and quadratic effects) using orthogonal contrast for equally spaced treatments estimated by the Proc GLM procedure of SAS. Data were presented as least-square means, with the standard error of the mean (SEM). For microbiome diversity analysis, statistical analysis was performed using a variety of computer packages, including XL stat, NCSS 2007, "R" and NCSS 2010. Alpha and beta diversity analysis was conducted as described previously [16,22] using Qiime 2 [6]. Significance reported for any analysis is defined as $P<0.05$.In addition, statistical comparisons of observed operational taxonomic units (OTUs); (Figure 3), Shannon Diversity indices (Figure 4), the principal coordinate plot of weighted UniFrac data **(**bacterial), and Bray-Curtis (fungi) (Figure 5) were conducted using Kruskal-Wallis pair wise comparisons.

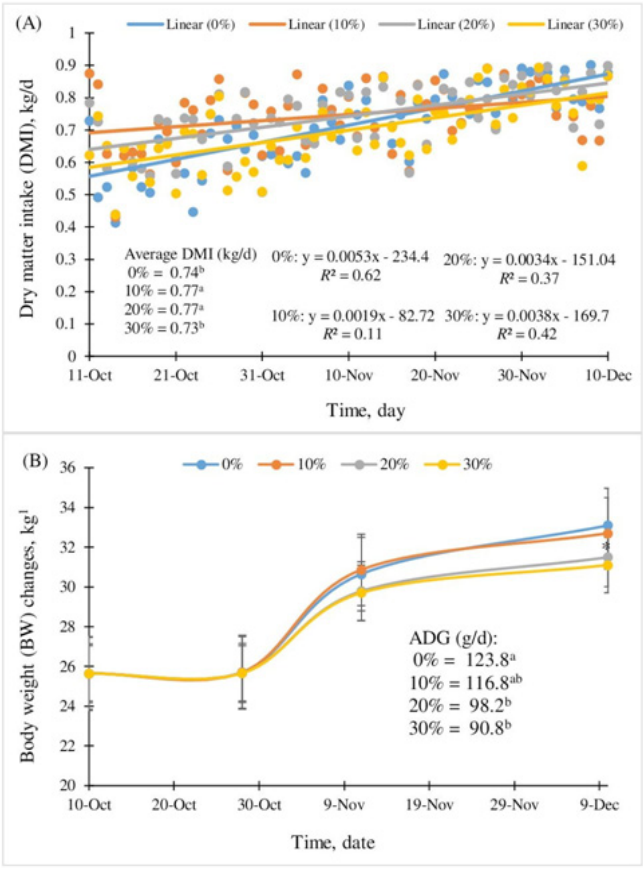

Figure 1: The effects of hemp seed meal (HSM) supplementation on mean dry matter intake (DMI; A) and body weight changes (B) of growing meat goats (n = 10). Results are the mean of each treatment, and error bars represent the standard error of the mean. Mean in a row with different letters (a, b) are different ($P< 0.05$).
*$P < 0.05$.
[1]Initial body weight (BW) from day 0 was used as a covariate.
ADG: Average Daily Gain; DMI: Dry Matter Intake.

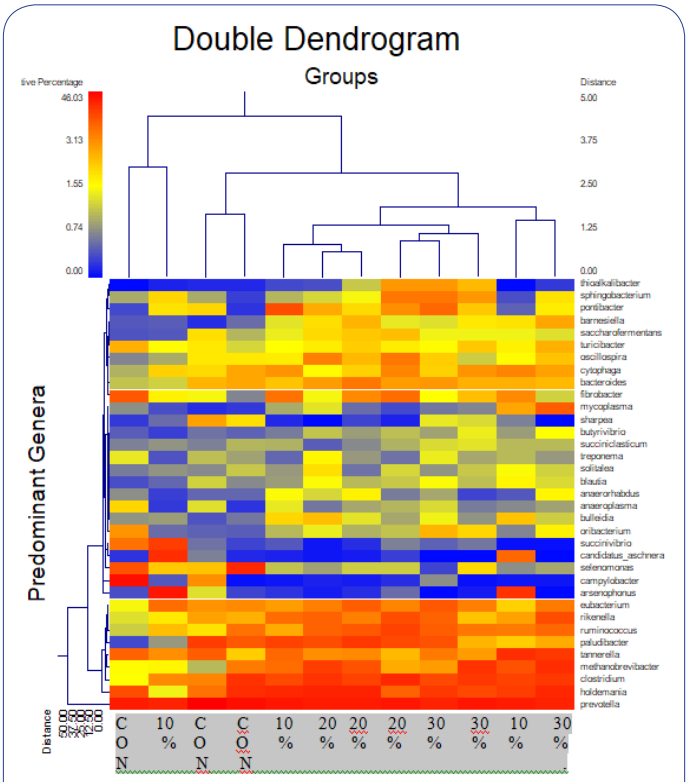

Figure 2: Dual Hierarchal dendrogram evaluation of the taxonomic classification of bacterial genera, with each sample clustered on the X-axis, labeled based upon the hemp seed meal (HSM) treatment. Samples with more similar microbial populations are mathematically clustered closer together. The genera (consortium) are used for clustering. Thus, the samples with a more similar consortium of genera cluster closer together with the length of connecting lines (top of heatmap) related to the similarity; shorter lines between two samples indicate a closely matched microbial consortium. The heatmap represents the relative percentages of each genus. The predominant genera are represented along the right Y-axis. The legend for the heatmap is provided in the upper left corner. Group: CON: Control.

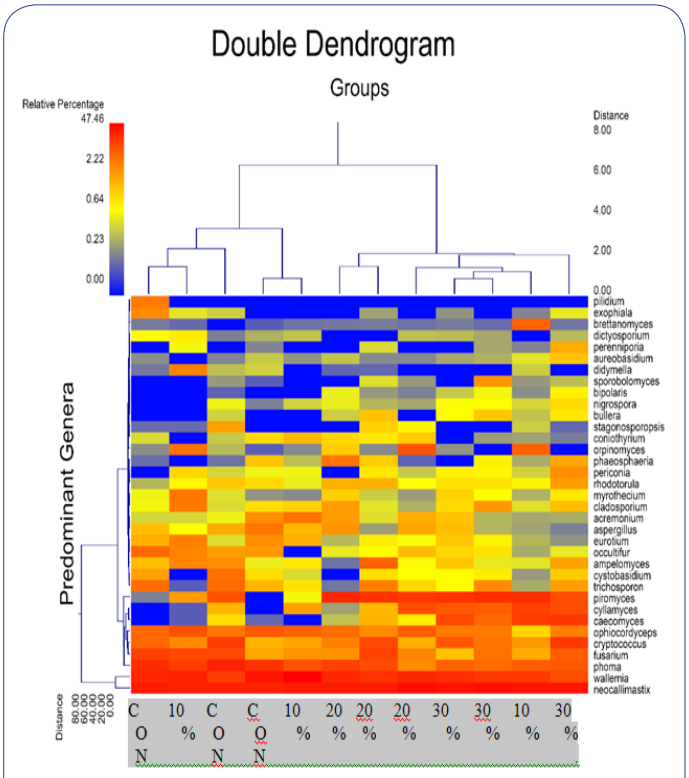

Figure 3: Dual Hierarchal dendrogram evaluation of the taxonomic classification of fungi genera, with each sample clustered on the X-axis, labeled based upon the hemp seed meal (HSM) treatment. Samples with more similar microbial populations are mathematically clustered closer together. The genera (consortium) are used for clustering. Thus, the samples with a more similar consortium of genera cluster closer together with the length of connecting lines (top of heatmap) related to the similarity; shorter lines between two samples indicate a closely matched microbial consortium. The heatmap represents the relative percentages of each genus. The predominant genera are represented along the right Y-axis. The legend for the heatmap is provided in the upper left corner. Group: CON: Control.

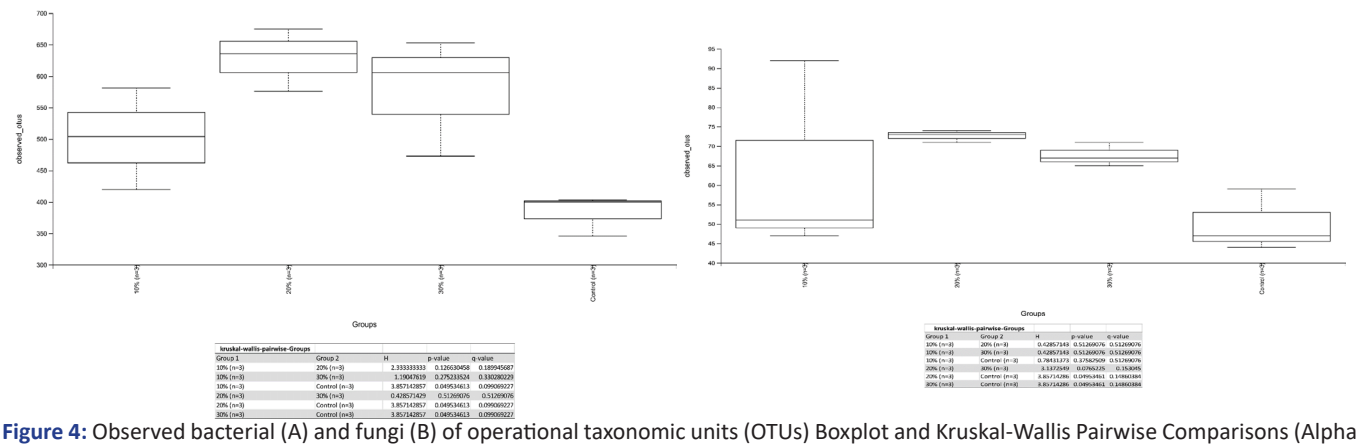

Figure 4: Observed bacterial (A) and fungi (B) of operational taxonomic units (OTUs) Boxplot and Kruskal-Wallis Pairwise Comparisons (Alpha diversity of samples) in meat goats fed hemp seed meal (HSM) supplementation.*P < 0.05.

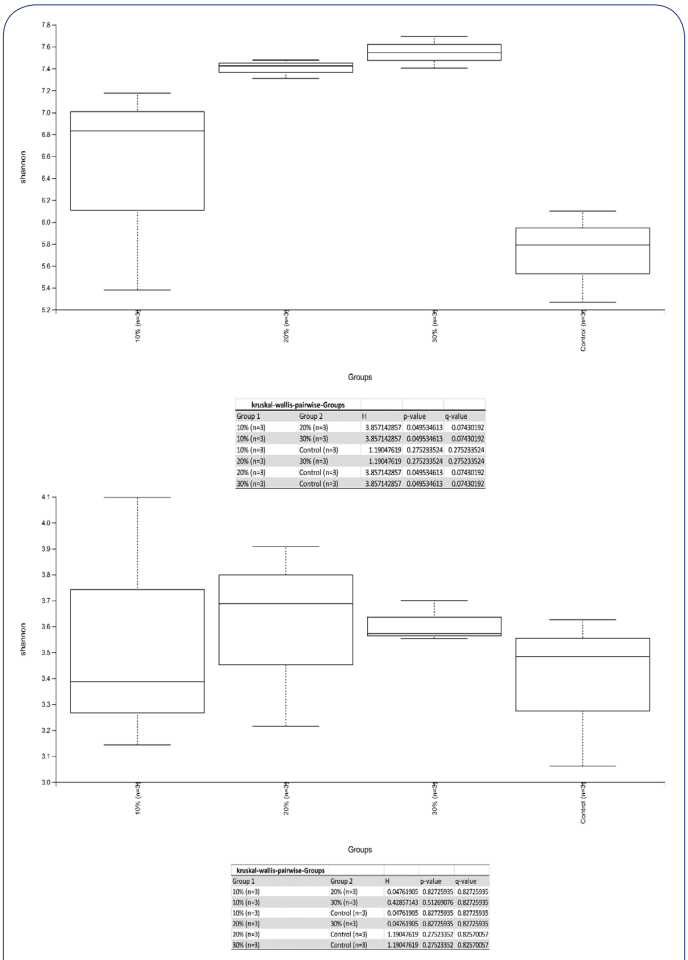

Figure 5: Shannon diversity of bacterial (A) and fungi (B) of operational taxonomic units (OUT) Boxplot and Kruskal-Wallis Pairwise Comparisons (Alpha diversity of samples) in meat goats fed hemp seed meal (HSM) supplementation.*$P < 0.05$

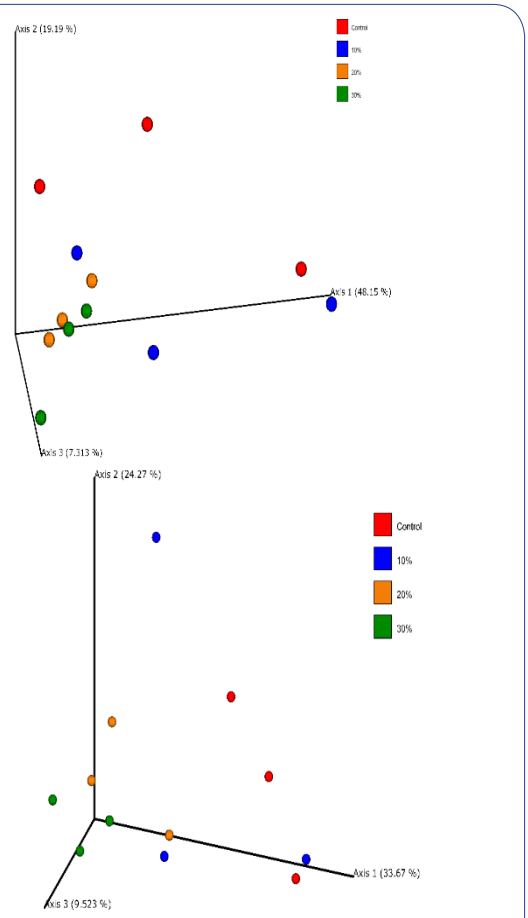

Figure 6: The principal coordinate plot of weighted Uni Frac data (Bacterial; A) and Bray-Curtis (Fungi; B) dissimilarity data (Beta diversity of samples) in meat goats fed hemp seed meal (HSM) supplementation.

Table 1: Mean diet formula and chemical composition of experimental diets and hemp seed meal (HSM) fed over the 60days feeding period to growing meat goats.

Ingredients	Hemp seed meal (HSM), % as-fed				HSM
	0	10	20	30	-
Timothy grass hay	50	50	50	50	-
Cracked corn	27	23	17.5	11.5	-
Soybean meal	19.5	14	9.0	5.0	-
Hemp seed meal (HSM)	0	10	20	30	-
Molasses	2.5	2.5	2.5	2.5	-
Goat premix	1	1	1	1	-
Chemical composition	---------- % dry matter (DM)----------				
Dry matter	89.1	88.7	89.1	89.4	89.6
Crude protein	19.2	19.9	19.3	20.4	36.4
Neutral detergent fiber	33.3	35.2	39.6	42.8	49.5
Acid detergent fiber	21.1	24.7	30.0	31.0	36.5
Acid hydrolysis fat	3.2	3.3	4.2	4.5	11.5
Lignin	3.3	4.8	6.2	7.0	12.3
Total digestible nutrient	71.2	69.2	64.7	62.8	63.2

HSM: Hemp Seed Meal.

Table 2: Mean relative abundance values (%) of the most predominant bacterial species (> 1.0%) as a function of the addition of hemp seed meal (HSM) supplementation with different dose levels of HSM in meat goats.

Bacterial species	Hemp seed meal (HSM) supplementation					
	0%	10%	20%	30%	SEM	*P* - value
Holdemania spp.	6.3	6.8	10.6	8.8	2.76	0.54
Mycoplasma spp.	0.8	1.0	0.5	1.7	0.77	0.19
Roseburia faecis	1.0^{a}	0.3^{b}	0.03^{b}	0.01^{b}	0.27	0.03
Prevotella ruminicola	26.0	13.8	14.6	14.2	4.99	0.12
Campylobacter spp.	11.9^{a}	0.09^{b}	0.03^{b}	0.14^{b}	3.36	0.05
Prevotella spp.	4.7	4.8	6.3	4.2	1.16	0.21
Selenomonas ruminantium	6.2^{a}	0.9^{b}	0.5^{b}	0.6^{b}	1.56	0.03
Clostridium spp.	4.5	4.6	8.6	7.0	1.57	0.10
Rikenella spp.	1.7	2.4	4.0	3.9	0.73	0.06
Eubacterium spp.	1.4	1.2	2.1	1.9	0.46	0.21
Tannerella spp.	3.3	5.5	2.5	4.7	1.55	0.27
Turicibacter spp.	1.4	1.2	1.5	1.8	0.26	0.16
Bacteroides spp.	1.4^{b}	1.3^{b}	2.4^{a}	2.0^{ab}	0.32	0.05
Fibrobacter succinogenes	1.9	2.5	2.5	1.3	0.79	0.10

[a-b]Means within row treatment with a different superscript differ at $P<0.05$. SEM: Standard Error of the Mean.
No linear or quadrate significant for the predominant bacterial species.

Table 3: Mean relative abundance values (%) of the most predominant bacterial phyla (> 1.0%) as a function of the addition of hemp seed meal (HSM) with different dose levels of HSM in meat goats.

Bacterial species	Hemp seed meal (HSM) supplementation					
	0%	10%	20%	30%	SEM	*P* - value
Bacteroidetes (B)	47.7^{ab}	43.0^{b}	48.0^{a}	45.4^{b}	6.32	0.01
Firmicutes (F)	32.4^{b}	28.6^{b}	44.5^{a}	42.7^{ab}	5.25	0.05
Proteobacteria	14.9	22.0	2.9	5.2	9.44	0.17
Fibrobacteres	1.9	2.5	2.5	1.3	0.79	0.61
Tenericutes	1.1	1.6	1.1	2.5	0.81	0.24
Spirochaetes	0.8	1.6	1.1	1.1	0.29	0.20
F/B ratio	0.67^{b}	0.67^{b}	0.93^{a}	0.94^{a}	0.045	0.05

$^{a-c}$ Means within row treatment with a different superscript differ at $P<0.05$. SEM = standard error of the mean.
No linear or quadrate significant for the predominant bacterial phylum.

Table 4: Mean relative abundance values (%) of the most predominant methanogen archaea (> 1.0%) as a function of the addition of hemp seed meal (HSM) supplementation with different dose levels of HSM in meat goats.

Bacterial species	Hemp seed meal (HSM) supplementation					
	0%	10%	20%	30%	SEM	*P* - value
Thermoplasma sp.	1.0^{ab}	1.3^{a}	0.4^{b}	0.3^{b}	0.19	0.01
	Linear: $P<0.05$. Quadrat: $P=0.11$					
Methanobrevibacter sp.	98.9^{ab}	98.5^{b}	99.4^{a}	99.5^{a}	0.22	0.01
	Linear: $P<0.05$. Quadrat: $P=0.34$					
Methanobacterium sp.	0.2	0.1	0.2	0.2	0.08	0.44

$^{a-b}$Means within row treatment with a different superscript differ at $P<0.05$. SEM: Standard Error of the Mean.

Results and discussion

Despite several decades of studies demonstrating the role of the ruminal and gastrointestinal microbial diversity in ruminants associated with bacterial and archaea populations, the response of the microbial consortium to feeding various levels of HSM diets remains largely unknown. Therefore, the principal objective of the current study was to assess the effects of HSM-containing diet supplementation as a feed replacement on rumen microbiome diversity changes in growing meat goats. The most significant findings in the present study were goats fed increasing levels of HSM, demonstrating a decrease ($P<0.01$) in the Bacteroidetes phylum (10 and 30% HSM) and non-archaeal methanogen *ThermoPlasma* sp. Populations. However, the abundance of Firmicutes, Firmicutes/Bacteroidetes ratio, and *Methanobrevibacter* sp. populations were increased ($P<0.05$) with increasing levels of HSM at 20 and 30%. There is no clear distinction between the treatment groups based on the bacterial clustering diversity, except the control and 10 % treatment. Conversely, based on the fungi clustering, there appears to be a distinction between the control, 10%, 20%, and 30% treatment groups.

Diet composition

The different proportions of HSM in the diets were chosen to provide a wide range of dietary fiber (NDF and ADF), total digestible nutrients, and fat to explore the dose-response effect. The mean CP concentration of treatment diets remained relatively steady at approximately 19%; however, the 30% treatment had a slightly elevated concentration CP (20.4%); (Table 1). Statistical comparisons were impossible because each feed ingredient was obtained from one source. The HSM used to formulate the diets in this experiment had a CP concentration of 36.4% DM. Variable CP concentrations are reported for HSM in the literature ranging from 31.9 to 38.5% [30,36,46]. 1). The HSM provided a high level of NDF, ADF, and lignin for goats having 49.5, 36.5, and 12.3% on DM basis, resulting in NDF, ADF, and lignin concentrations increasing in the experimental diets with increasing levels of HSM (Table 1) which is similar to the reported by Mustafa [30].

Intake and animal performance

The intake potential of a feedstuff is a crucial element. For example, despite high levels of fiber (e.g., NDF and lignin) and fat in HSM, the inclusion of 10 and 20% increased ($P<0.05$) DMI compared to control and 30% HSM supplementation (Figure 1A) indicated that HSM supplementation from 10-20% did not have any detrimental effects on DMI in goats meat. A similar study by Karlsson showed that lambs fed with HSC, peas, rapeseed cake, or the control diet exhibited no differences in DMI [36]. This finding is also in agreement with other studies that found no adverse effects on DMI of feeding HSM [46], full-fat hempseed [27], or HSC [30] in lambs, cattle, and dairy cows, respectively. Hempseed cake, conversely, has a low DMI, rumen DM digestibility, and total volatile fatty acids (VFA) production when compared to canola meal or soybean meal in steers and sheep [61,62].

Previous studies have shown that feeding WHS or HSC supplementation did not affect the final live weight or ADG in steers [27], growing cattle [30], and dairy cows [36]. Conversely, in the present study, the BW growth and ADG significantly decreased ($P<0.05$) with increasing levels of HSM at 20 and 30% after covariate by initial BW (Figure 1B). This could be reflected in lower DMI in the diet at 30% HSM supplementation than the control diet. Similar results have been reported for growing cattle [30] when animals received HSM compared with a soybean meal diet. Furthermore, diets containing moderate levels of HSC (143 g/kg DM), corresponding to a dietary CP concentration of 157 g/kg DM, resulted in the maximum yields of milk and energy-corrected milk by dairy cows compared to high levels of HSC (233 g and 318 g/kg DM) or control group (0 g/kg DM) [36]. These results are consistent with the findings in other studies that have shown that there is no further improvement in milk yield and animal performance when increasing the dietary HSC (>143 g/kg DM) or HSM [28,36,51]. Their research indicates that increasing the proportion of HSM in the growing meat goat diets results in curvilinear responses concerning DMI with a simultaneously affected animal performance.

Bacteria, archaea, and fungi diversity

After stringent quality sequence curation, in the present study, 786,404 sequences were parsed, and 688,553 were clustered. 688,546 sequences identified within the Bacteria and Archaea domains were utilized for final microbiota analyses. The average reads per sample were 57,378. For alpha and beta

diversity analysis, samples were rarefied to 18,000 sequences. Data were evaluated in a multivariate manner to determine the changes between groups. However, after stringent quality sequence curation, 511,766 sequences were parsed, and 463,079 were clustered. 462,629 sequences identified within the Fungi domain were utilized for final microbiota analyses. The average reads per sample were 38,552.

To provide a visual overview combined with the analysis, we utilize a dual hierarchal dendrogram to display the predominant genera with clustering related to the different groups. For example, based on the bacterial clustering seen in (Figure 2), control and 10% HSM treatment groups appear to trend closer toward one another when compared to the remaining treatment. Still, there is no clear distinction between the treatment groups. Conversely, based on the fungi clustering evident in (Figure 3), there appears to be a distinction between both the control and 10% treatment groups and the clusters in 20% and 30% treatment groups.

Alpha diversity is an ecology term that refers to the diversity within a specific area or ecosystem and is usually expressed by the number of species (i.e., species richness) in that ecosystem. The number of OTU at the species level can be evaluated to define alpha diversity among different groups. In the present study, statistical comparisons of observed OTUs and Shannon diversity indices for each sample group in bacterial (Figure 4A and 5A) and fungi (Figure 4B and Figure 5B) were conducted using Kruskal-Wallis pairwise comparisons. Based on the number of observed OTUs (Figure 4A), the bacterial diversity detected within the control treatment group is significantly less (P<0.05) than the diversity detected within the three HSM treatment groups. However, analysis of the Shannon diversity (Figure 5A) indices between experimental groups, which account for species evenness and species richness, detected no significant difference between the control and 10% treatment groups (P=0.275). Both the control and 10% treatment groups did display a significantly less (P<0.05) diverse environment when compared to the 20% and 30% treatment groups. Based on the number of observed OTUs (Figure 4B), the fungi diversity detected within the control treatment group is significantly less (P<0.05) than the diversity seen within the 10% and 20% treatment groups. However, analysis of the Shannon diversity (Figure 5B) indices between experimental groups, which accounts for species evenness in addition to species richness, detected no significant difference between the 4 experimental treatment groups, indicating that the HSM treatment did not adversely affect the overall bacterial and fungi species richness of the rumen microflora of the growing meat goats.

Beta diversity is an analysis of the microbial community structure. This analysis is performed by creating individual phylogenetic trees and then statistically evaluating each tree. The microbial community structure was analyzed using weighted UniFrac distance matrices (Bacterial) and Bray-Curtis dissimilarity matrix (fungi) in the present study. Principal coordinate analysis plots were used to visualize the data in these matrices and determine any significant differences between the microbial communities. In (Figure 6A), there appears to be no phylogenetic assemblage amongst any treatment group that is significantly different from the remaining groups. However, in (Figure 6B), there seems to be a phylogenetic assemblage amongst the control treatment group that differed (P=0.09) from the 20% and 30% treatment groups in terms of fungal diversity. The primary vector explains the 48.1% bacterial diversity (Figure 6A). The first 3 vectors together exhibit 74.6% of the variation among the groups. For fungi diversity (Figure 6B), the primary vector explains 33.6% of the divergence between the groups. The first 3 vectors together exhibit 67.4% variation among the fungi groups. These data suggest that exposure to HSM supplementation affected the rumen bacteria and fungi diversity in the rumen of meat goats.

Predominant microbial community and methanogens diversity changes

More than 512 bacterial species (excluding unknown species) were classified from the ruminal contents of the goats in this study. However, only the 14 most abundant bacterial species (>1.0%) are presented in (Table 2). This agrees with the data of the Hungate project, which reported a considerably larger number of bacterial species (501 genomes; 480 bacteria and 21 archaea) [64] with access to bacterial cultures (http://www.rmgnetwork.org/hungate1000.html). The populations of *Prevotella ruminicola* (26%), *CamPylobacter* spp. (11.9%), *Holdemania* spp. (6.3%), and *Clostridium* spp. (4.5%) were relatively the most abundant species in the control diet. Even though an increased level (P<0.05) of *Bacteroides* spp. has been found in the rumen of goats fed with HSM supplementation, the relative decrease in *Roseburia faecis* (P<0.03), *Cam Pylobacter* sp. (P<0.05), and *Selenomonas ruminantium* (P< 0.03) populations in the meat goats, indicates that these microbial populations may be dependent upon the increased HSM levels in the rumen compared to control diet. Altering from a grain-based diet (e.g., high starch) to a high forage-based (e.g., high fiber) diet induces large alterations in the microbial composition of the rumen to adjust from metabolizing primarily starch to cellulose [48]. For example, the genera Butyrivibrio and Fibrobacter are cellulolytic bacteria and thus are more abundant when the diet is high in plant fiber [48]. Conversely, the genera Selenomonas, Ruminobacter, and Eubacterium are more abundant when the diet is grain-based. Thus, shifts in the abundance of these genera of bacteria are noted when changing from forage-based diets to grain-based diets. However, there was no clear distinction in the changes in abundance of these types of bacteria in this study, suggesting that the HSM treatment did not cause a change in microbial populations degrading cellulose and starch nutrients.

Our initial goal in this study was to determine whether there were any relationships between the bacterial communities inhabiting the rumen of host animals when HSM was included. Generally, 20 phyla were identified, but only 6 were recognized in meat goats above the 1.0% threshold (Table 5). Among the major 6 bacterial phyla, the populations of Bacteroidetes (47.7%), Firmicutes (32.4%), and Proteobacteria (14.9%) were relatively the most abundant bacterial phylum across the diets (Table 5), as demonstrated in previous studies [47,48]. An earlier study with Japanese native goat (*CaPra aegagrus hircus*) fed diets containing 50% Timothy grass and 50% concentrate diets showed an abundance of Bacteroidetes and Firmicutes phylum (60 and 24%, respectively; [14], which supports the results of the present study. In the present study, however, Bacteroidetes was lower (P<0.01) in animals fed 10 and 30% HSM. At the same time, the relative abundance of Firmicutes and Firmicutes/Bacteroidetes ratio was greater (P<0.05) in the rumen of goats fed 20 and 30% HSM than in those consuming the control diet (Table 5). Results from the current studies indicated that a decrease in the proportion of sequences assigned to the Bacteroidetes phylum was observed for the increasing supplements of HSM with a concomitant increase in Firmicutes. Bacteroidetes phy-

lumis more abundantin the rumen of Holstein dairy cows fed with 30% roughage plus 70% concentrate diets [32]. However, the current study showed the rumen of growing meat goats displayed a more significant proportion of Firmicutes (Table 5) and Firmicutes/Bacteroidetes ratio compared to Bacteroidetes phylum across HSM diets. This finding agrees with the data of [26]. They reported a considerably larger number of Firmicutes populations detected in animals fed grass hay-based (prairie hay) diet in beef steers. Likewise, [13] provided a pasture-based diet to cannulated steers and found a significant increase in Firmicutes populations compared to total mixed ration (TMR) grain-based diets. However, the bacterial community composition was also associated with diets, gender, and time [55,74].

Lessening enteric CH_4 emissions from ruminants while improving feed conversion efficiency and dietary nutrient utilization aims for sustainable livestock industries. Up to 28 genera and 113 species of methanogens have been recognized to exist in nature [33], and five of these species belong to *Methanobrevibacter* and *Methanosarcina* genera. Both *Methanobrevibacter ruminantium* and *Methanomicrobium mobile* have been identified as the major methanogens in the rumen [31,42,43,70,73]. In comparison, [14,33] reported that *Methanobrevibacter* was considered the dominant methanogens in the rumen (61.6%), which supports the results of the present study (Table 4). In the present study, the abundance of *Methanobrevibacter* sp. increased (linear; $P<0.05$) as HSM increased in the diets, while non-methanogenic archaea, *ThermoPlasma* sp., was reduced (Linear; $P<0.05$) by 60 to 70% with 20 and 30% HSM inclusion in the diets (Table 4). This study indicated that animals consuming HSM might potentially increase/or decrease CH_4 productions in the rumen of meat goats, which may have impacted the energy efficiency and animal performance in growing meat goats [34]. It has been stated that there is a positive correlation between CH_4 production and a higher abundance of *Methanobrevibacter* species and bacterial community structure in dairy cows [11,12] and in sheep and goats [43]. However, [68] reported that whole hempseed was 8% more effective at reducing CH_4 than linseed oil. [18] Documented a 10% decrease in CH_4 production for hempseed oil compared to corn oil. Lower CH_4production is most likely due to the high polyunsaturated fatty acids (e.g., α-linolenic and linoleic acids) content in hempseed oil; that plays a role by inhibiting protozoa activity and acting as hydrogen sink through biohydrogenation [54,68]. However, whole hempseed, hemp bark, hem steam, and hemp extractives are more effective than oil at inhibiting methanogens. Since these parts have more terpenes, polyphenols, essential oils, and lignans that are more toxic to methanogens than polyunsaturated fatty acids [41,53], these compounds accumulate in cytoplasmic membranes as they are lipophilic, thus disrupting methanogen cell membranes [40,41]. Many cyclic hydrocarbons, e.g., aromatics, cycloalkanes, and terpenes, are toxic to microorganisms and alter the cytoplasmic membrane [65]. The resulting changes in the membrane structure affect the membrane-embedded proteins and the impediment function of the membrane, which results in the depreciation of metabolic activity [65]. Therefore, reducing CH_4 production might maintain or improve animal performance by conserving energy redirected to animal growth [34,35]. The limited *in-vivo* studies on the anti-methanogenic effects of hemp by-products on animal performance warrant further research.

Implications

Recently, hemp was a renewal source as a plant that offers a wide variety of applications (textile, pharmaceuticals, construction, etc.), including also the use in animal and human nutrition. Therefore, moderate levels of HSM supplementation (10-20% as-fed) could be a good feeding strategy to maintain or improve the DMI, microbiome community compositions, and animal performance in meat goats: the high levels of crude proteins and fat in the HSM and its by-products could be used in meat goats as dietary supplementation in feed mainly as sources of essential fatty acids and crude protein. However, up to now, few publications do not allow to suggest the optimal dosage of the co-products for the different ruminant species. In addition, no experiments have been published on the use of whole plants as forage for ruminants. Nevertheless, the high protein content, amino acid profile, and unsaturated fatty acids of HSM and hemp's by-products seem exciting and suitable for ruminant nutrition. Analysis of the number of OTU at the species level and Shannon diversity indices between experimental groups, which accounts for species evenness in addition to species richness, detected no significant difference between the three experimental treatment groups, except for 30% HSM inclusion, indicating that the optimal levels of HSM treatment (<20% HSM) did not adversely affect the overall bacterial and fungi species richness of the rumen microflora of the growing meat goats. However, the high level of HSM supplementation, up to 20% HSM (as-fed basis), may have affected rumen fermentation profiles and animal performance by altering rumen microbiota and warrants further research for its effects on rumen metabolites associated with microbiome community composition.

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