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RESEARCH ARTICLE

Rumen Microbial Community and Functions of Rumen Bacteria under Different Feeding Regime

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ABSTRACT

The aim of the study was to evaluate the variations in the rumen ecosystem at genus level and predicted functions of rumen bacteria by using whole crop corn silage (WCS), whole crop rice silage (WRS) or rice straw (RS) as forage sources in beef cattle ration. Ruminal digesta samples from 10 bulls per treatment were collected at day 60 of experimental period. The PCoA plots based on the Bray-Curtis distance matrix (BDM) expressed separation between WRS and WCS , WRS and RS using PC1 ($P<0.05$, 45.56%). The PCoA plots based on BDM also expressed separation among WRS, WCS and RS group using PC2 ($P<0.05$, 11.95%). Microbiota composition resutls at genus level showed that the most abundant genera were *Prevotella* (13.37%) and *Ruminococcus* (4.00%). Comparison of treatments represented that *Prevotella, Treponema* and *Anaerostipes* were higher in bulls fed WCS forage. *Clostridium, Anaeroplasma* and *RFN20* were higher in bulls fed RS forage than animals fed WCS and WRS. *Butyrivibrio* was higher in WRS and RS treatments than WCS treatment group. *Pseudobutyrivibrio* was higher in bulls fed WRS forage than animals fed RS and WCS forage. *Fibrobacter* was higher in RS and WCS as compared to WRS. The results of functional alteration of rumen microbiota in different experimental groups represented that the leading modified function of the microbiome was the transporter. Based on findings of current study, it is concluded that microbial community at genus level in the rumen of bulls was highly altered by forage type.

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INTRODUCTION

In Europe and developed countries, silage of different forages or grasses are being used for beef cattle production (Lengowski *et al.*, 2016). In contrast, different agricultural by products like corn stover, rice straw, wheat straw, corn stalk and corn stovers are being used in under develop and developing countries for fattening of beef cattle. It has been reported that beef cattle rations composition influences the intake, ruminal fermentation, and ruminal microorganism communities (Paz *et al.*, 2018; Qiu *et al.*, 2020) and hence animal productivity (Chen *et al.*, 2019). However previous studies reported that feeding forages in the form of hay, silages, and crop residues influence intake of nutrient, growth, rumen microbiota and ruminal fermentation parameters variably (Niu *et al.*, 2017; Chen *et al.*, 2019). For instance, in the study of Owens *et al.* (2009), silage type did not influence the ruminal pH, while in the experiment of Abrahamse *et al.* (2008) ruminal pH was reduced with corn silage. Similarly, previous studies also reported inconsistent results on rumen volatile fatty acids parameters due to the types of forage (Abrahamse *et al.*, 2008; Owens *et al.*, 2009; Brask *et al.*, 2013).

In last decade, most of *in vitro* experiments were conducted to assess the influence of silages type and agricultural by product types on the ruminal microbiota (Witzig *et al.*, 2010; Witzig *et al.*, 2015; Liu *et al.*, 2016). *In vitro* experiments explored that type of silage (grass silage vs corn silage) not only alter the abundance of *Bacteroides-Prevotella* and *Firmicutes* (Witzig *et al.*, 2010; Witzig *et al.*, 2015) but results in change in microorganisms' composition (Lengowski *et al.*, 2016). Similarly, in another *in vitro* study, it has been reported that forage type (rice straw and alfalfa hay) had impact on rumen microbiota and in abundances of dominant genera *Butyrivibrio, Anaeroplasma*, *Prevotella* and *Fibrobacter*

(Liu *et al.,* 2016). Similar with *in vitro* studies, *in vivo* studies also supported the narrative thtat silage type influence the rumen microbial composition. For example, in an *in vivo* study (Lengowski *et al.,* 2016), it has been observed that abundance of rumen bacteria and *Fibrobacter succinogenes* enhanced in rumen digesta (solid portion) of corn silage fed cows. Lengowski *et al.* (2016) also reported that *Selenomonas ruminantium* and *F. succinogenes* abundance increase in the liquid portion of rumen digesta of corn silage fed cows, while *Prevotella*, *Ruminobacter,* and *Ruminococci* abundance increase in both solid and liquid portion of digesta of corn silage fed cows.

Previous studies review has provided the evidence that type of silage and type of forage influence the rumen microbial population variably *in vitro* and in dairy cows. Therefore, a study was required to investigate influence of forage type product and silage type on rumen microbial composition in beef animals. The aim of this investigation was to investigate the variation in ruminal microbiota composition due to whole crop corn silage (WCS), rice crop corn silage (RS) and rice straw (RS) in hybrid angus bulls. The emphasis of the current experiment was mainly to check the impact of WCS, WRS and RS on the ruminal microbial composition at genus level and prediction of ruminal bacterial function *in vivo*. We hypothesized that forage type would differently affect the temporal fluctuations of bacterial composition at genus level and hence predicted function of rumen bacterial species in-vivo.

MATERIALS AND METHODS

Experimental design, animal management and feed: In the current *in vivo* experiment, a total 30 of Angus hybrid bulls was used. The body weight of the experimental bulls was 272.43±21.80 kg. Experimental bulls were divided into three group and each group had 10 animals. Group one received *ad libitum* WCS while a 1.75 Kg concentrate allowance per bull per day was ensured. Group two received WRS *ad libitum* while a 1.75 Kg concentrate allowance per bull per day was ensured. Group three received RS *ad libitum* while a 1.75 Kg concentrate allowance per bull per day was ensured. All other managemental conditions for all experimental animals were same throughout the experimental trial. Animals were provided fresh 24 hours round the clock. Animals were reared in stress free environment.

Sample collection: At day 60 of the experiment after the onset of feeding experimental diets, ~100 mL of bulls rumen liquid (RL) including digesta were collected by passing the mouth tube into rumen. After collection of RL, upper ~50 mL of RL was discarded and remaining ~50 mL of RL was safely stored at -20°C for ruminal bacterial composition by 16S rRNA analysis.

DNA extraction and 16S rRNA pyrosequencing: From the RL samples, DNA extraction and 16S rRNA pyrosequencing was carried out by using standard method as presented in the previous study with some modification (Chen *et al.,* 2020). In short, a total of 1.5 mL of rumen fluid sample was centrifuged at 1000 × g for 15 minutes. After centrifugation, sediment was eradicated. Clear supernatant extract was obtained by 2nd centrifugation at 12000 × g for 15 minutes. For extraction of DNA from the supernatant, a commercial kit was used, and Qubit 3.0 was used to quantify DNA. For the amplification of bacterial 16S rRNA genes at V3-V4 area from isolated DNA, barcoded primers were used. Then, Illumina MiSeq platform was applied to purify the PCR products after preliminary check of size and specificity via agarose gel electrophoresis. Lastly, Illumina MiSeq platform (San Diego, CA, U.S.A) was used for high-throughput sequencing following the manufactures protocol.

Pyrosequencing data analyses: To filter the raw reads (RR) and to remove low quality seq QIIME (Version 1.9.1) was used. FLASH (Version 1.2.7) was used to merge the filtered data into tags. QIIME (Version 1.9.1) was also used to identify the merged the seq with high quality. UCHIME algorithm was applied in U Search software (Version 8.1.1861) for the removal of chimeric tags. Clustered tags of each sample into operational taxonomic units (OTUs) at 97% similarity was obtained by using UCLUST algorithm. Furthermore, illustrative seq for each OTUs were selected and annotated for the taxonomic information. A principal coordinate analysis (PCoA) based on the weighted UniFrac distances was performed. Permutational multivariate analysis of variance was performed by R (Version 2.1.5.3) to evaluate differences among groups.

Wilcoxon ranks sum test was carried out to evaluate the differential abundance of genera and software R (Version 3.3.3). Out of all genera, the genera with an adjusted P value <0.05 were assumed significant. To predict metagenomic function, PICRUST (Langille *et al*., 2013) was used. In short, operational taxonomic units (OTUs) were selected from a demultiplexed fasta file covering the seq for all subjects employing the closed reference method, against the Green Genes reference database. These OTUs were normalized by the predicted 16s copy number and functions were predicted from these normalized OTUs with the help of Green Genes database for KEGG Orthologs. From this, abundances of the predicted metagenomic function for every single sample was achieved.

Statistical analyses: The microbiome data of RL were statistically analyzed with the GLM procedure by using SPSS. Statistical differences between the experimental treatments were stated at $P < 0.05$. The differences between experimental diets at $0.05 \leq P \leq 0.10$ were supposed to have a tendency for significance.

RESULTS

Illumina seq: 10 samples from each group was used to generate the RR by Illumina MiSeq PE250 sequencing. Overall, a total of 30 samples from three groups were used to generate the RR by Illumina MiSeq PE250 sequencing. After Illumina MiSeq PE250 sequencing, a total of 1,758,219 high quality joined reads were obtained. A total 57,276 raw Tags were attained with an average of 48,031 effective Tags per sample, with an average length of 410 bp were assigned to 2,474 operational OTUs of ruminal bacterial base on a 97 similarity cut-off.

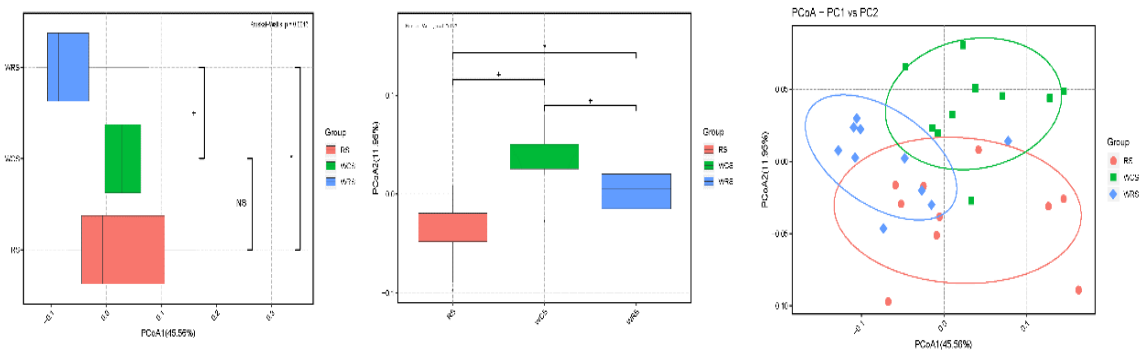

Fig. 1: PCoA of the bacterial communities from different treatments. The greater the distance between two points, the lower the similarity between them, whereas samples with more similar bacterial communities cluster closer together.

Table 1: Genera composition (Major Genera) of the rumen bacteria influenced by different forage source

Phylum	Genus	Relative abundance (%)				SEM	P
		All	WRS	RS	WCS		
Bacteroidetes	*Prevotella*	13.37	11.35^{b}	13.36^{b}	15.57^{a}	1.219	0.008
	CF231	1.26	1.10^{b}	1.50^{a}	1.27^{a}	0.116	0.015
	YRC22	1.15	0.89	1.10	1.35	0.133	0.285
	BF311	0.08	0.04^{b}	0.20^{a}	0.1^{a}	0.047	0.000
Firmicutes	*Ruminococcus*	4.00	3.98	3.70	4.55	0.250	0.105
	Succiniclasticum	3.20	3.06	3.07	3.53	0.155	0.230
	Clostridium	1.08	1.05^{b}	1.33^{a}	0.92^{b}	0.121	0.012
	Butyrivibrio	0.82	0.89^{a}	0.91^{a}	0.71^{b}	0.064	0.031
	Pseudobutyrivibrio	0.41	0.59^{a}	0.31^{b}	0.42^{b}	0.081	0.065
	Coprococcus	0.74	0.77	0.68	0.83	0.044	0.410
	RFN20	0.34	0.23^{b}	0.42^{a}	0.25^{b}	0.060	0.084
	Moryella	0.18	0.20^{b}	0.07^{c}	0.29^{a}	0.064	0.000
	Dehalobacterium	0.19	0.23	0.16	0.17	0.022	0.733
	Oscillospira	0.16	0.20	0.16	0.16	0.013	0.532
	Anaerostipes	0.08	0.07^{b}	0.08^{b}	0.14^{a}	0.022	0.039
	Mogibacterium	0.12	0.13	0.12	0.09	0.012	0.224
	p-75-a5	0.08	0.10^{a}	0.06^{b}	0.08^{b}	0.012	0.034

Mean values in the same row with different letters differ significantly: RS, Rice straw; WCS, whole crop corn silage; WRS, whole crop rice silage: Standard error of mean.

Table 2: Genera composition (Minor Genera) of the rumen bacteria influenced by different forage source

Phylum	Genus	Relative abundance (%)				SEM	P
		All	WRS	RS	WCS		
Spirochaetes	*Treponema*	0.59	0.40^{b}	0.48^{b}	0.78^{a}	0.116	0.010
Fibrobacteres	*Fibrobacter*	0.89	0.30^{b}	0.89^{a}	1.73^{a}	0.415	0.001
Tenericutes	*Anaeroplasma*	0.27	0.22^{b}	0.45^{a}	0.21^{b}	0.078	0.010
	Moraxella	0.04	0.09	0.17	0.03	0.041	0.337
Proteobacteria	*Desulfovibrio*	0.13	0.14^{a}	0.12^{b}	0.14^{a}	0.007	0.048
Euryarchaeota	*Methanobrevibacter*	0.08	0.16	0.06	0.08	0.031	0.098
Chloroflexi	*SHD-231*	0.08	0.14^{a}	0.10^{b}	0.07^{c}	0.020	0.007

Mean values in the same row with different letters differ significantly: RS, Rice straw; WCS, whole crop corn silage; WRS, whole crop rice silage: Standard error of mean.

Diversities of rumen microbiota: Analysis of the beta diversity is presented in PCoA Fig. 1. The PCoA plots based on BDM expressed separation between WRS and WCS, WRS and RS by using PC1 (P<0.05, 45.56%). The PCoA plots based on the BDM also expressed separation among WRS, WCS and RS group by using PC2 (P<0.05, 11.95%).

Rumen bacteria composition at Genus level: The analysis of the microbial composition down to the genus level (Table 1 and Table 2) revealed that the most abundant genus were *Prevotella* (13.37%) and *Ruminococcus* (4.00%), followed by *Succiniclasticum* (3.2%), *CF231* (1.26%), *YRC22* (1.15%), *Clostridium* (1.08%), *Fibrobacter* (0.89%), *Butyrivibrio* (0.82), *Coprococcus* (0.74%), *Treponema* (0.59%), *Pseudobutyrivibrio* (0.41%). The relative abundance of the top 10 rumen mibriobita genera is presented in Fig. 1.

Table 1 and 2 represent the relative abundance ≥0.1% of rumen microbita genera in all rumen fluid collected samples. Results of rumen microbiota at the genus level represented that *Prevotella*, *CF231*, *BF311*, *Clostridium*, *Butyrivibrio*, *Pseudobutyrivibrio*, *RFN20*, *Moryella*, *Anaerostipes*, *p-75-a5*, *Treponema*, *Fibrobacter*, *Anaeroplasma*, *Desulfovibrio*, *Methanobrevibacter*, and *SHD-231* were significantly different among the experimental groups excluding minor genera, such as *YRC22*, *Ruminococcus*, *Succiniclasticum*, *Coprococcus*, *Dehalobacterium*, *Oscillospira*, *Mogibacterium* and *Moraxella* (Table 1 and 2). Comparison of treatments represented that *Prevotella* presenece at genus level was more in WCS group incontrast to other experimental diets. Genus level comparison expolred that *CF231* presence was lower in WRS fed animals than animals reared on WCS and RS forage. Similary, at genus level. *BF311* presence was less in animals reared on WRS treatment than animals reared on WCS and RS forage. *Clostridium* presence at genus level was more in animals fed RS forage than animals reared on WCS and WRS. Simlarly, the presence of *Butyrivibrio* at genus level was more in WRS and RS fed animals than animals fed WCS.

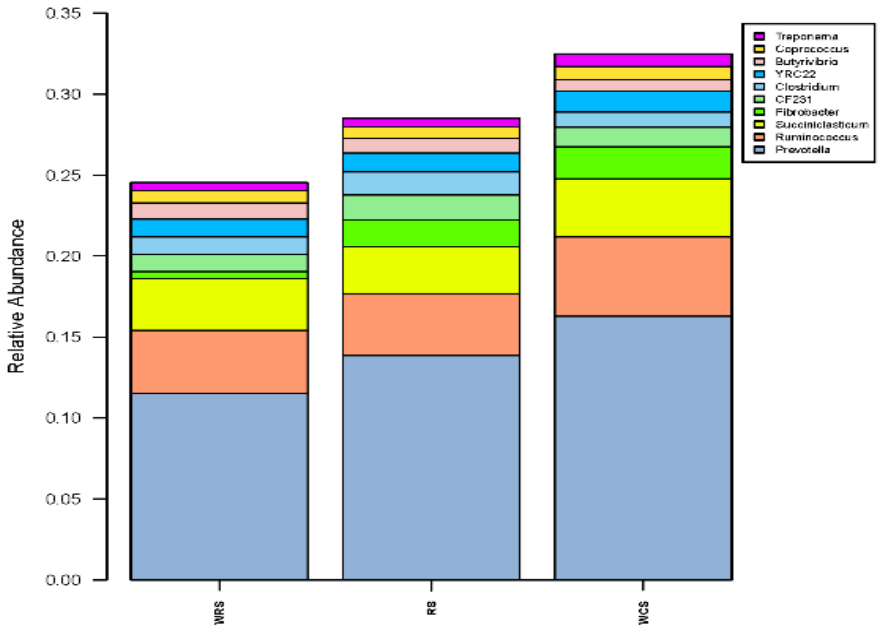

Fig 2: The relative abundance of rumen bacteria at the genus level. Each bar represents the relative abundance of each sample. Each color represents a genus.

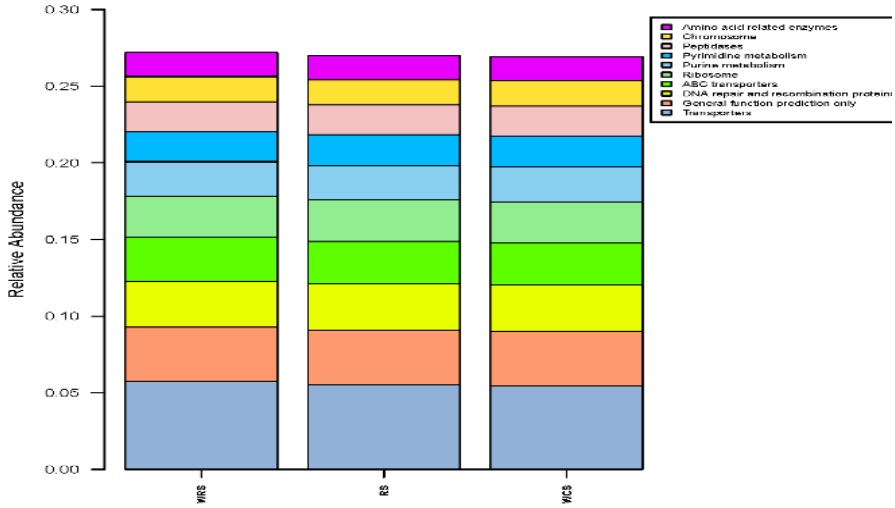

Fig. 3: Predictive function analysis (Top 10 pathway-Level 3).

At genus level, *Pseudobutyrivibrio* presence was more in the RL samples of the animals reared on WRS forage than animals reared on RS and WCS forage. *RFN20* was higher in animals fed RS based diet than animals reared on WCS and WRS diet. Comparison of treatments represented that *Moryella* was higher in WCS treatment and lowest *Moryella* was observed in RS. *Anaerostipes* species at genus level was more in animals reared on WCS forage than animals reared on RS and WRS forage. *p-75-a5* was higher in WRS as compared RS and WCS. *Treponema* at genus level was more in animals reared on WCS forage than animals reared on WRS and RS forage. *Fibrobacter* was higher in RS and WCS as compare to WRS. *Anaeroplasma* also at genus level was higher in animals reared on RS forage than animals reared on WCS and WRS treatment. *Desulfovibrio,* at genus level was more in animals fed RS based diet and WCS based diet than animals fed RS forage based diet. *SHD-231* was higher in WRS and WCS as compared to RS.

Predicted Functions of Bacteria Related to different dietary treatments on growing beef cattle: Predicted functions of Bacteria related to different dietary treatments on growing beef cattle represents that the most abundant function was linked to genetic information, processing and metabolism in the rumen microbial population and their analyses identified altered microbiota functions at category level three in the experimental treatments (Fig. 3). In all the experimental treatments, the prominant altered functions of the microbiota was the transporter. Similarly, within bulls, bacterial OTUs identified feed efficiency models that were predicted to have functiona l categories associated to general rumen function and protein related metabolism (DNA repair and recombination, ribosome, purine metabolism, peptidases, pyrimidine metabolism, and amino acid related enzymes).

DISCUSSION

Diversity metrics are being used to evaluate the species evenness and richness in a specific sample (Tucker *et al.*, 2017) and could effectivley be used in rumen fluid samples. In the current study, ruminal fluid samples from bulls reared on RS forage-based diet showed higher microbial

diversity than animals fed WRS and WCS forage. These findings are supported by the previous researcher (Qiu *et al.*, 2019), and the reported that animals reared on highly fermentable carbohydrates (CHO)-based diet show decreased microbial diversity. The differences in microbial diversity may be supported by the previous established theory that rumen bacterial diversity is highly influenced by ruminal pH (Lv *et al.*, 2020). Previous researcher, Abrahamse *et al.* (2008), reported that silage feeding resulted in lower ruminal pH and lower microbial diversity. In the current study, silage feeding resulted in lower microbial diversity as compared to RS treatment. These findings are in line with earlier report (Kim *et al.*, 2016). Kim *et al.* (2016) reproted that animals reared on diet contained high fermentable CHO decreased microbial diversity.

Microbiota composition analysis down to the genus level reveleaed that the most abundant taxa were *Prevotella*, *Ruminococcus*, *Succiniclasticum*, *CF231*, *YRC22*, *Clostridium*, *Butyrivibrio*, *Coprococcus,* and *Pseudobutyrivibrio* and belonged to abundant phylum except *Fibrobacter* and *Treponema* that belonged to minor phylum *Spirochaetes* and *Fibrobacteres.* In this experiment, *Prevotella* accounted 13.37% of the total rumen microbial population and was the most dominant genus. The abundance of *Prevotella* is major contributor of protein metabolism in rumen, especially oligopeptides breakdown in the rumen. The higher abundance of *Prevotella* in WCS as compared to other treatments represents higher protein degradation in animals on WCS treatments. *Prevotella* in the rumen of ruminants is the most dominant ruminal microbiota specie in bulls fed both high fermentable carbohydrate-based diet and forage based ration (Niu *et al.*, 2017) which is similar with the findings of our experiment. *Ruminococcus* was the second most abundant genus in this experiment. It has been reported that *Ruminococcus flavefaciens* and *Ruminobacter albums* can degrade large amounts of cellulose and hemicellulose from fiber (Purushe *et al.*, 2010). However, in this experiment, the second most abundant genus *Ruminococcus* was similar in all experimental treatments representing most of the fiber degradation was similar in all the experimental treatments. The genus *Succiniclasticum*, which contributed 3.20% of the total bacterial population, is known to involve in fermentation process of the rumen and help in the conversion of volatile fatty acids especially conversion of succinate into propionate (Gylswyk, 1995).

Finding of the study explored that *Butyrivibrio* accounts 0.82% of the total microbiota population in bulls. It has been reported that *Butyrivibrio* produce mucosal butyrate and release butyrate and enhance the availability of volatile fatty acid butyrate for the animals (Baldwin *et al.*, 2012). The lower concentration of *Butyrivibrio* in rumen of bulls fed WCS represent low absorbability of volatile butyrate from rumen wall that may influence the growth of fattening bulls. It has been reported that *Fibrobacteres* are key cellulolytic bacteria in ruminants (Loor *et al.*, 2016). *Fibrobacteres* results showed that seq of *Fibrobacteres* were only 0.89% of the total rumen bacterial population, which was in line with earlier reports in ruminants (Zened *et al.*, 2013; Niu *et al.*, 2017). The diet of animals reared on RS forage contained more structural CHO had higher abundance of *Fibrobacteres.* Similar findings have been reported by Qiu *et al.*, (2020) who stated that structural CHO rich diet had more *Fibrobacteres* abundance. The genus *Treponema*, commonly present in the rumen of the ruminants, participate in degrading soluble fibre (Qiu *et al.*, 2020). Previous studies reported that WCS have higher concentration of soluble fiber (Chen *et al.*, 2019), therefore higher *Treponema* presence in rumen of animals fed WCS was expected in our study. The *Proteobacteria* phylum abundance in rumen is relatively low, however it performs vital role in the rumen metabolism especially in animals fed nonstructural CHO rations (Qiu *et al.*, 2020). In the current study, *Desulfovibrio* genera belongs to phylum *Proteobacteria* showed higher abundance in bulls fed WRS and WCS diets contained higher contents of nonstructural CHO. Our results are similar with the reports of earlier researcher who stated that the genera of phylum *Proteobacteria* are abundant in animals fed nonstructural CHO rations (Qiu *et al.*, 2020). The genus *SHD-231* belongs to Phylum *Chloroflexi* and *Chloroflexi* higher level has been seen in goat's cecal microbial population reared on diets contained grains and higher fermentable CHO. Same findings have also been reported in high milk producing cows by Derakhshani *et al.* (2017). In the present experiment, *SHD-231* higher abundance in animals reared on WCS and WRS could be explained by the presence of higher fermetable carbohydates WCS and WRS diet.

In the present study, a greater part of the operational taxonomic units found through the regression models among all experimental treatments were anticipated to have transporters in high numbers. Similarly, in the recent study, Paz *et al.* (2018) predicted higher number of transporters in steers fed both energy dense ration and forage. In ruminants production systems, feed efficacy is significantly alterd by the potential of the rumen microbiota to fetch feed energy and the ability of the rumen microbiota to yield rumen microflor protein as a high true and crude protein source for animal. Nevertheless, the projection of *transporters* higher number in the current study was surprising. Popova *et al.* (2017) reported that higher abundance of transporters mediates nutrient uptake, hence higher number of transporters in the current study suggest important role of transporters in ruminant's performance fed different forages.

Conclusion: The importance of the rumen microbiota for feed digestion is well known in the rumen of the ruminants. Current experiment results explored that rumen microbial population at genus level was highly altered by forage type in bulls. This study also discovered a subset of bacterial OTUs that could have influence on feed efficiency in bulls reared on different forages.

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Authors contribution: DC and QT conceptualize the experiment. DC, HZ and KC managed bulls, collected rumen samples, and analyzed the rumen samples. SH and GZ conducted DNA extraction. SH performed 16S rRNA pyrosequencing and pyrosequencing data analyses.

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