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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 rice crop 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 expressed separation between WRS and WCS , WRS and RS using PC1 ($P<0.05$, 45.56%). The PCoA plots based on the Bray-Curtis distance matrix 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 genus were *Prevotella* (13.37%) and *Ruminococcus* (4.00%). Comparison of treatments represented that *Prevotella*, *Treponema* and *Anaerostipes* were higher in WCS group as compared to other treatments. *Clostridium*, *Anaeroplasma* and *RFN20* was higher in RS group as compared to other treatments. *Butyrivibrio* was higher in WRS and RS treatments as compared to WCS. *Pseudobutyrivibrio* was higher in WRS treatment as compared to RS and WCS. *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, corn silage or silage of different grasses is being used for beef cattle production (Lengowski *et al.*, 2016). In contrast, crop residues like rice straw, corn stover, corn stalks and wheat straw are being used in developing countries for fattening cattle as forage source. 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). Effect of feeding silages, hay or crop residues on intake, growth, rumen microbial population and ruminal fermentation parameters was inconsistent in previous experiments (Niu *et al.*, 2017; Chen *et al.*, 2019). For example, in the study of Brask *et al.* (2013) and Owens *et al.* (2009), pH was not affected by silage type, while the ruminal pH was decreased with corn silage in the study of Abrahamse *et al.* (2008). In addition, variable results on production of volatile fatty acids were observed in various studies depending on the types of forage fed to ruminants (Owens *et al.*, 2009; Brask *et al.*, 2013).

In last decade, most of invitro studies in past were conducted to assess the effect of type of silages and agricultural by products (like wheat straw, rice straw, corn stover) on the ruminal microorganism (Witzig *et al.*, 2010; Witzig *et al.*, 2015; Liu *et al.*, 2016). Invitro studies reported change in abundance of *Firmicutes* and *Bacteroides-Prevotella* (Witzig *et al.*, 2010; Witzig *et al.*, 2015) and alterations in microorganisms' composition when incubating grass silage instead of corn silage (Witzig *et al.*, 2015; Lengowski *et al.*, 2016). Similarly, in-vitro studies conducted by Liu *et al.* (2016) reported a significant differences in abundances of dominant genera *Butyrivibrio*, *Anaeroplasma*, *Prevotella* and *Fibrobacter*

between rice straw and alfalfa hay forage type. In in-vivo study of Lengowski *et al.* (2016), total bacteria abundance and increase in *Fibrobacter succinogenes* was observed in solids fraction of rumen digesta of dairy cows fed corn silage. They further reported that grass silage feeding to dairy cow increases *Selenomonas ruminantium* and *F. succinogenes* abundance in the liquid portion of rumen digesta and higher abundance of *Ruminobacter amylophilus, Prevotella bryantii, and ruminococci* in both solid and liquid portion of digesta.

Based on review it is clear that type of silages and forage type have variable influence on ruminal microorganisms' community, and consequently on fermentation parameters. In the current study we used, rice straw, whole crop rice silage and whole crop corn silage in the diet of bulls because rice straw is abundant, cheap and is the major forage source for animals in the tropical zones of the world and other two forages are recognized for its better quality and is used globally as forage in ruminant production. Therefore, this study was planned to further investigate the ruminal microbiota variation by using whole crop rice silage (WCS), whole rice crop silage (WRS) or rice straw (RS) as forage source in the feed of bulls. The focus of the study was mainly to check the impact of WCS, WRS and RS on the ruminal bacterial composition at genus level and prediction of ruminal bacterial function in vivo. We hypothesized that WCS, WRS and RS would differently effect 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 diet: In the current study, a total 30 Angus hybrid bulls (body weight = 272.43±21.80 kg) were divided into three groups in such a way that each group received one of three dietary treatments. The dietary treatments were: WCS, whole-plant corn silage diet; WRS, whole plant rice silage and RS, rice straw diet. A 1.75 Kg of concentrate per day per cattle was offered. Silages were offered *adlibitum*.

Sample collection: At day 60 of the experiment after the onset of feeding experimental diets, nearly 100 mL of rumen digesta sample were orally collected by using mouth tube. After collection nearly ~50 mL of rumen liquid was stored at −20℃ for ruminal bacterial composition by 16S rRNA analysis.

Chemical analytical procedures: In the current study, chemical composition of experimental diets was determined using the standard procedure of AOAC.

DNA extraction and 16S rRNA pyrosequencing: The procedure used for DNA extraction as well as 16S rRNA pyrosequencing is fully explained in our published work (Chen *et al.*, 2020). In brief, 1.5. mL of ruminal fluid sample was centrifuged at 1000 × g for 15 minutes. After eradicating sediment of centrifuged rumen sample, the clear supernatant extract was eliminated by second centrifugation at 12000 × g for 15 minutes. A commercial kit was used to extract the DNA and Qubit 3.0 was used to quantify DNA. Barcoded primers were used to amplify bacteria 16S. rRNA. genes of the V4.-.V3 area from isolated DNA. PCR were conducted in triplicate. Then, Illumina MiSeq platform was applied to purify the PCR products after preliminary check of size and specificity via agaroose gel electrophoresis. Lastly, Illumina MiSeq platform (San Diego, CA, USA) was used for high-throughput sequencing following the manufactures protocol.

Pyrosequencing data analyses: The detailed procedure of pyrosequencing data analyses is given in our published study (Chen *et al*., 2020). QIIME (Version 1.9.1) was used to filter the raw reads and to remove low quality sequences. FLASH (Version 1.2.7) was used to merg the filtered data into tags. Furthermore, the merged sequences with high quality were identified by QIIME. Moreover, for removal of chimeric tags, the Uchime algorithm was applied in U.search software (Version .8.1.1861.). Clustered tags of each sample into operational taxonomic units (OTUs) at 97% similarity was obtained by using U.clust algorithm. Furthermore, illustrative sequence for each OTUs were selected and annotated for the taxonomic information. A principal coordinate analysis (PCoA) based on the weighted Uni.Frac distances was performed to compare all collected samples. 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 considered significant. To predict metagenomic function, PICRUSt (Langille *et al*., 2013) was used. In short, OTUs. were selected from a demultiplexed fasta. file covering the sequences. for all subjects. employing the closed reference method, against the Green.Genes reference. database. These OTUs. were normalized. by the predicted 16s. copy no and functions. were predicted from these normalized. OTUs. with the help of Green.Genes database for KEGG. Orthologs. From this, abundances. The predicted. metagenomic function for every single sample was achieved.

Statistical analyses: The microbiome data were analyzed with the general linear model procedure in SPSS Version 18. Statistical differences were stated at P.<0.05. Differences between experimental treatments at 0.05≤ P≤0.10 were supposed to have a tendency for significance.

RESULTS

Illumina sequence: A total of 30 samples from three groups were used to generate the Raw reads by Illumina MiSeq PE250 sequencing. Quality trimming, pair-end joining, and chimeric filtering was used for downstream analyses of raw reads to obtain a total. of 1,758,219 high quality. joined reads. A total 57,276. raw Tags were attained with. an average of. 48,031 effective. Tags per. sample (Supplementary table. 1), with an. average. length. of 410 bp. were. assigned. to 2,474 operational OTUs. of ruminal bacterial. base on a 97 similarity cut-off.

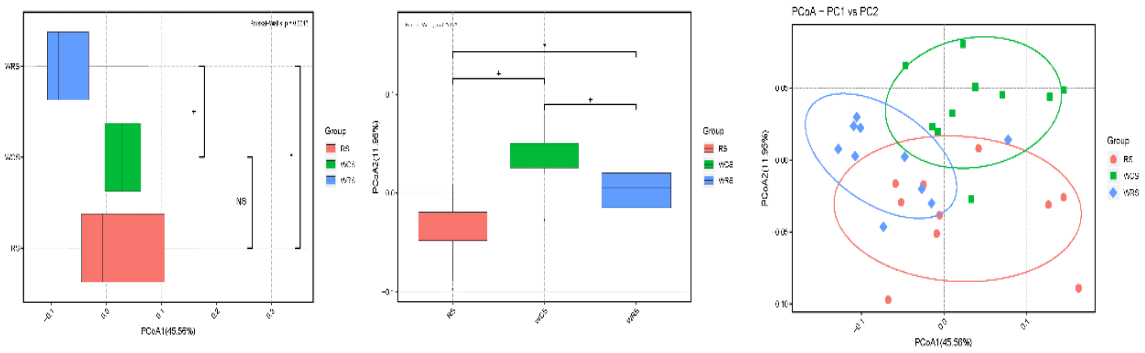

Fig. 1: PCoA of the bacterial communities from different treatments. The greater the distance between two points, the lower the similarity between them, whereas samples with more similar bacterial communities cluster closer together.

Table 1: Genera composition (Abundant phylum) of the rumen bacteria influenced by different forage source in rumen of bulls

Phylum	Genus	Relative abundance (%)				SEM	P
		All	WRS	RS	WCS		
	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
Bacteroidetes	*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
	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
Firmicutes	*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 (a, b, c) differ (P<0.05): [a]RS, Rice straw; WCS, whole crop corn silage; WRS, whole crop rice silage: [b]Standard error of mean.

Table 2: Genera composition (Minor phylum) of the rumen bacteria influenced by different forage source in rumen of bulls (relative abundance ≥0.1%)

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 (a, b, c) differ (P<0.05): [a]RS, Rice straw; WCS, whole crop corn silage; WRS, whole crop rice silage: [b]Standard error of mean.

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

Rumen bacteria composition at Genus level: The analyze 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. genera of rumen. is presented in Fig 1.

Table. 1 and. 2 represents the relative abundance ≥0.1% of genera in. all. rumen fluid samples. At. the. genus. level, *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 except for some. minor. genera, such as *YRC22*, *Ruminococcus*, *Succiniclasticum*, *Coprococcus*, *Dehalobacterium*, *Oscillospira*, *Mogibacterium* and *Moraxella* (Table 1 and Table 2). Comparison of treatments represented that *Prevotella* was higher in WCS group as compared to other treatments. *CF231* was lower in WRS treatment as compared to WCS and RS treatments. Similary, *BF311* was lower in WRS treatment as compared to WCS and RS treatments. *Clostridium* was higher in RS group as compared to other two treatments. *Butyrivibrio* was higher in WRS and RS treatments as compared to WCS.

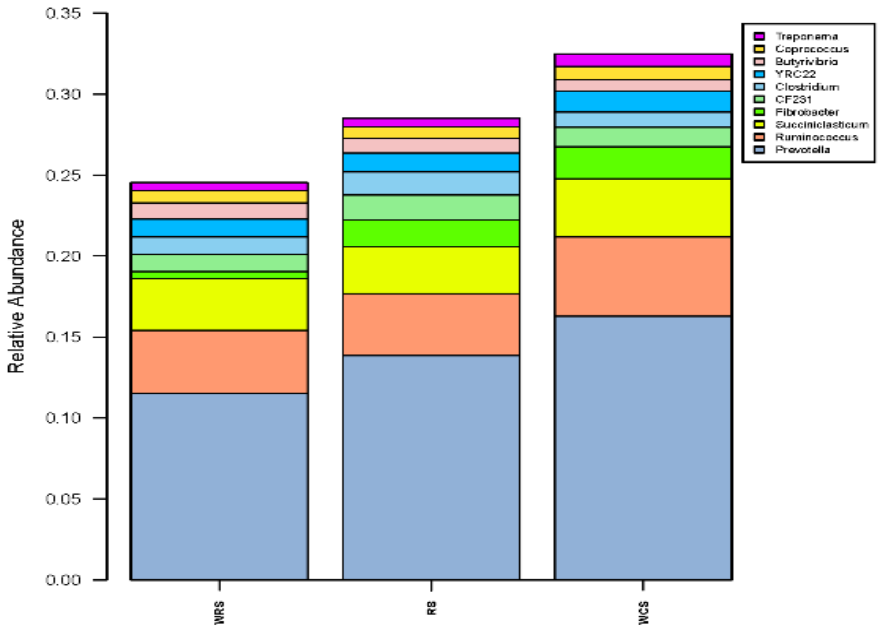

Fig 2: Bacterial community structure variation in different stages. The relative abundance species of bacteria at the genus level is shown. Each bar represents the relative abundance of each sample. Each color represents a genus.

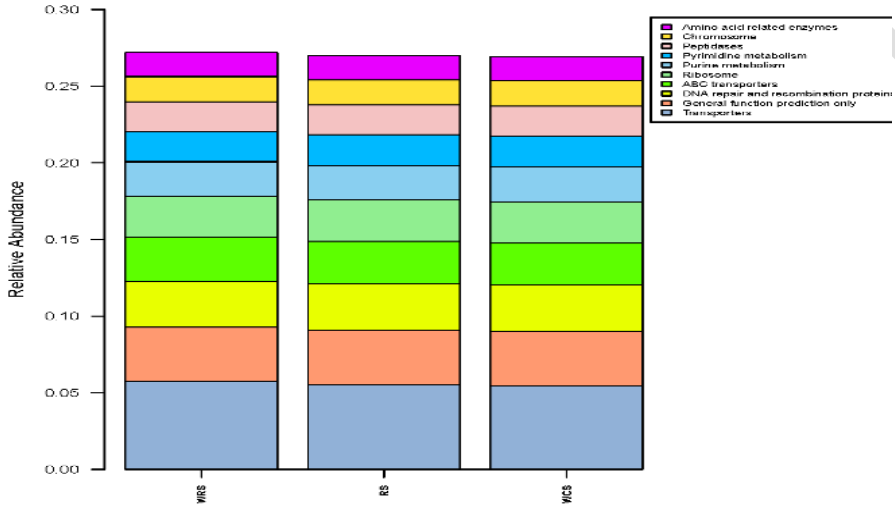

Fig. 3: Predictive function analysis (Top 10 pathway-Level 3).

Pseudobutyrivibrio was higher in WRS treatment as. compared. to RS and WCS. *RFN20* was higher in RS as compared to WCS and WRS. Comparison of treatments represented that *Moryella* was higher in WCS treatment and lowest *Moryella* was observed in RS. *Anaerostipes* was higher in WCS as compared to other treatments. *p-75-a5* was higher in WRS as compared RS and WCS. *Treponema* was higher in WCS as compared to WRS and RS. *Fibrobacter* was higher in RS and WCS as compare to WRS. *Anaeroplasma* was higher in RS as compare to WCS and WRS. *Desulfovibrio* was higher in WCS and RS as compared to RS. *SHD-231* was higher in WRS and WCS as compared to RS.

Predicted Functions of Bacteria Attached to different dietary treatments on growing beef cattle: The most. abundant gene. function. was linked. to Genetic. Information. Processing. and metabolism. in the. rumen microbial population (Supplement Table 4). Further. analyses identified. altered microbiota functions. at category. level. 3 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, peptidases, ribosome, pyrimidine. metabolism, purine. metabolism, and amino. acid related. enzymes).

DISCUSSION

It has been reported that diversity. metrics. are being. used to evaluate the species. richness. and evenness. in a specific sample. (Tucker *et al.*, 2017). In the current study, ruminal fluid samples from bulls fed RS diet showed higher microbial diversity as compared to two types of silage, which is similar with many reports (Qiu *et al.*, 2019; Qiu *et al.*, 2020) in which highly fermentable carbohydrates-based diet decreased microbial diversity. Similar results have also been reported by Qiu *et al.* (2020). These differences in microbial diversity may be supported by the well-established. notion that ruminal.

pH. has a significant impact. on the rumen bacterial. diversity (Lv *et al.,* 2020). In the current study, rumen. samples. from bulls fed silages showed lower ruminal pH (supplementary table 2) and lower microbial diversity as compared to RS fed animals that had higher ruminal pH. These findings are in line. with. earlier report (Kim *et al.,* 2016) in which grain-based diets that contained high fermentable carbohydrates 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, the moost dominant. genus. *Prevotella* in the rumen of bulls accounting 13.37% of the total bacterial population. 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. Previous studies reported that *Prevotella* is a predominant ruminal microbiota specie in bulls. fed. both. forage. and grain (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 fermenting. succinate. and transforming it into propionate. (Gylswyk, 1995).

Finding of this 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 improve. the bioavailability. of butyrate. for the host (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. *Fibrobacteres* are major cellulolytic. bacteria. in the. rumen of ruminants (Zened *et al.,* 2013). Results. of. current. experiment found that the sequences of *Fibrobacteres* were only 0.89% of the total bacterial population, which was similar to an earlier reports in ruminates (Zened *et al.,* 2013; Niu *et al.,* 2017). In current study, RS diet had more structural carbohydrates and represents higher abundance of *Fibrobacteres* which is similar with the findings of Qiu *et al.,* 2020 who reported that fiber rich diet had higher abundance of *Fibrobacteres* in the rumen of steers fed high fibrous diet. 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 fibre (Chen *et al.,* 2019), therefore higher *Treponema* presence in rumen of animals fed WCS was expected in the current experiment. *Anaeroplasma* genra belongs to Phylum *Tenericutes* and It has been reported that abundance of *Tenericutes* reduced at higher rate because of their intolerance to low rumen pH (Loor *et al.,* 2016). In this experiment the decrease in *Anaeroplasma* in WCS and WRS could be attributed to lower rumen pH linked with silage as reported by earlier researcher (Abrahamse *et al.,* 2008). The *Proteobacteria* phylum abundance in rumen is relatively low, however it performs vital role in the rumen metabolism especially in animals fed nonstructural carbohydrates rations (Qiu *et al.,* 2019; 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 carbohydrates. The results of current study are similar with the reports of previous researcher who reported that the genra of phylum *Proteobacteria* are abundant in animals fed nonstructural carbohydrates rations (Qiu *et al.,* 2019; Qiu *et al.,* 2020). The genus *SHD-231* belongs to Phylum *Chloroflexi* and a higher abundance of *Chloroflexi* has been reported in goat's cecal microbial population fed diets with higher amount of fermentable carbohydrates. Similar results has been reported by Derakhshani *et al.* (2017) in dairy cows. In the current study, higher abundance of *SHD-231* in WCS and WRS could be justified by higher fermetable carbohydates in WCS and WRS as compare to RS diet of the bulls.

In the current study, a greater part of the operational taxonomic units found through the regression models among all experimental treatments were predicted to have large number of transporters. 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 influenced by the potential of the rumen microbiota to extract energy from the feed and the ability of the rumen microbiota to yield microbial cell protein as a protein source for the host ruminant. Nevertheless, the prediction of higher number of transporters 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 study findings revealed that microbial community 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, H Z and KC handled experimental animals, collected samples, and analyze the samples for fermentation parameters. SH and GZ carried out DNA extraction and 16S Rrna pyrosequencing and pyrosequencing data analyses.

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