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RESEARCH ARTICLE

Supplementation of Laminaria Japonica Powder Influence Ruminal Microbiota Without Affecting Ruminal Fermentation in Bulls

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ABSTRACT

The impact of supplementation of Laminaria japonica powder on ruminal microbiota composition and fermentation parameters was evaluated in the present paper. For this purpose, a total of 30 cross bred Angus bulls (Xiangxi Yellow Cattle ♀ x Angus ♂, body weight = 331.22 ± 10.15 kg) were equally divided into two groups (n= 15). Two experimental diets were prepared that were basal diet (CON) and basal diet supplemented with 0.7% Laminaria japonica powder commonly known as kelp powder (SWE). The trial period was 60 days. The results showed that the addition of Laminaria japonica powder to the diet had no effect on rumen fermentation parameters in bulls (P>0.05). Results of microbial compositions explored that at both phylum and genus levels, most abundant phylum was Bacteroidetes (59.82%) and Firmicutes (29.75%). Results also showed that most abundant taxa were *Prevotella* (35.04%) and *Ruminococcus* (7.52%), and at the genus level 37.68% of the sequences were unclassified. The results of the relative abundance of the top 10 genera of rumen showed that *Succiniclasticum*, *Moryella*, and *Comamonas* were significantly different in ruminal bacteria relative abundance between the two experimental treatments (P<0.05). There was a notable distinction between CON and SWE cohorts using PC2 (P<0.01, 16.66%). Based on results, it is concluded that Laminaria japonica powder influence rumen microbial composition.

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INTRODUCTION

Since 1960, seaweeds macroalgae have gained attention for their rich nutrient content as animal feed (McHugh, 2002). In many parts of the world, seaweeds are cultured due to its rich nutritional profile like and bioactive compounds like proteins, peptides, amino acids, mineral, and polysaccharides (Cornish *et al.*, 2020). There are more than 1000 classes of seaweeds, of which the main genera and species that can be used as animal feed are *Laminaria species*, *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Lithothamnion species*, *Sargassum species Ulva species*, and *Palmaria palmata* (Guiry and Guiry, 2013; Makkar *et al.*, 2016). Numerous studies have shown that seaweeds can be used as feed for livestock (Makkar *et al.*, 2016; Clemmons *et al.*, 2019; Roque *et al.*, 2019; Xue *et al.*, 2019; Abbott *et al.*, 2020) while Makkar *et al.* (2016) reported seaweeds contain many bioactive compounds that can be used as prebiotics to improve the performance of ruminants (Makkar *et al.*, 2016).

Laminaria japonica known as Kelp is one of the algae of the *Laminaria species*, which contains bioactive compounds with various health benefiting effects including prebiotic, antioxidant, antimicrobial, anti-inflammatory, immunomodulatory and anticancer effects (Overton and Yasui, 2014; Yu *et al.*, 2018). Throughout recent decades, numerous research efforts have demonstrated the beneficial effects of using seaweeds in animal feed to promote livestock health and performance (Ananthi *et al.*, 2010; Abudabos *et al.*, 2013; Ahmed *et al.*, 2022; Anderson *et al.*, 2023). However, fewer studies have been conducted on the *Laminaria japonica* feeding in ruminants and its impact on ruminal permeation pattern and ruminal microbiota composition. The ability of ruminants to degrade and digest

unutilizable roughage lies mainly in the microbiome in the rumen, where alterations in the nutritional profile of the feed affect the rumen microbial composition, and similarly, changes in the rumen microbial composition lead to intermediate and end products produced by rumen fermentation and digestion of feed (Gençoğlu and Türkmen, 2006; Pitta *et al.*, 2016). Therefore, in this study, we chose to add *Laminaria japonica* as a supplement to finishing bulls feed to investigate the *Laminaria japonica* powder as a feed additive on rumen flora and rumen fermentation parameters of finishing bulls.

MATERIALS AND METHODS

All procedure and protocol carried out in the current study were approved by Animal Care and Use Committee of Hunan Agricultural University, Changsha, China (Permission No. 2020011).

Materials Experimental design, animal management, and diet: The experiment was carried at Xinhuang Xiaofeiniu Food Co., Ltd. (Huaihua City, Hunan, China). The kelp powder was provided by Rongcheng Hongde Marine Bio-Tech Ltd., Shandong, China. A total of 30 Angus hybrid bulls (Xiangxi Yellow Cattle ♀ x Angus ♂, body weight = 331.22 ± 10.15 kg) were divided into two groups (n= 15) and fed the basal diet (CON) or basal diet supplemented with 0.7% (DM basisv) kelp powder (SWE). A total 14 days period was given as adoption period to adjust new diet. The total duration of experiment was 60 days. All animals were fed single pen with 3 kg of concentrate per bull per day. Animals were ensured *ad-libitum* access to roughage and water. The chemical composition of the experimental feeds as well as the concentrate compositional is shown in Table 1.

Table 1: Composition and nutrient levels of experimental diets (% DM basis).

Items	Dietary treatment		Distiller's grain	Soybean curb residue
	CON[2]	SWE[3]		
Ingredients %				
Distiller's grain	53.94	53.94		
Soybean curb residue	16.18	16.18		
Corn	22.41	22.41		
Wheat bran	2.09	2.09		
Cottonseed meal	0.60	0.60		
Rapeseed meal	0.60	0.60		
Soybean meal	0.90	0.90		
Sesame meal	0.60	0.60		
Amino acid dregs	0.90	0.90		
Puffing urea	0.30	0.30		
Premix[1]	1.48	1.27		
kelp powder	0.00	0.21		
Total	100.00	100.00		
Nutrient level %				
Organic matter	82.05	81.87	87.22	93.66
Dry matter	64.21	65.36	41.86	20.54
Crude protein	18.32	18.4	14.76	18.3
Ether extract	5.04	5.34	3.29	1.57
Neutral detergent fiber	31.87	31.95	41.49	39.67
Acid detergent fiber	18.09	18.14	22.86	28.48

[1] Every kilogram of mineral–vitamin premix contained the following: 160 g $NaHCO_3$; 8000 IU Vitamin A, 1200 IU Vitamin D3, 40 IU Vitamin E, 15.85 mg Cu, 88.68 mg Fe, 63.05 mg Mn, 59.99 mg Zn, 9.04 mg Mg, 0.21 mg Se, 0.83 mg I, 0.16 mg. [2] control group. [3] kelp powder group.

Sample collection: On the 40th day of the trial, a rumen sampler was employed to extract ruminal fluid via the oesophagus 3 h post-morning feed. Ruminal fluid was then strained through four layers of cheesecloth (250 μm) to obtain pure ruminal fluid and to remove any foreign particles as described in recent study (Yue *et al.*, 2023). Collected ruminal fluid was divided two equal prats and one part was used for analyses of pH, ruminal VFAs, and NH3-N. The content of VFA was determined by gas chromatograph (GC-2010plus, Shimadzu, Japan) following the protocol of previous studies (Muetzel *et al.*, 2011; Wilk *et al.*, 2022) and NH_3-N was determined by phenol-sodium hypochlorite colorimetric method (T/CAAA003-2018). The second part was stored in liquid nitrogen immediately after adding the stabilizer and then stored at −80 ◦C for DNA extraction and 16S rRNA analysis. A portable pH meter (SI400, Spectrum Technologies, Inc., America) was used to measure the pH of each sample immediately. The quantity of each VFA and total VFAs (TVFA) in the aliquots of ruminal fluid were determined by gas chromatograph (GC-2010, Shimadzu, Kyoto, Japan).

DNA extraction and 16S rRNA pyrosequencing: Stored ruminal fluid for DNA extraction and 16S rRNA analysis were taken and processed for DNA extraction and 16S rRNA analysis. After removal from storage, ruminal fluid was thawed and homogenised and the DNA of homogenized ruminal fluid was extracted using the Magen Hipure Soil DNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The samples of DNA were quantified using a Qubit 3.0 Fluorometer (Q33216, invitrogen, USA). After extraction of bacterial 16S rRNA, genes of the V3-V4 region were amplified from the extracted DNA employing custom barcoded primers (5′- CCTACGGRRBGCASCAGKVRVGAAT-3′) and (5′-GGACTACNVGGGTWTCTAATCC-3′). The PCR products were subsequently examined for size and specificity via agarose gel electrophoresis and purified. Finally, high-throughput sequencing was conducted using the Illumina MiSeq platform (San Diego, CA, USA) following the manufacturer's protocol. To remove low quality sequence, raw reads were filtered using QIIME (version 1.9.1) (Caporaso *et al.*, 2010) to remove low-quality sequences. After that, filtered data were merged into tags by FLASH (Magoc and Salzberg, 2011) (Version 1.2.7) and the merged sequences with high quality were identified by QIIME and used for further analysis. Then, the Uchime algorithm in Usearch software (Version 8.1.1861) was applied to remove chimeric tags. The resulting tags for each sample were clustered into operational taxonomic units (OTUs) at the level of 97% similarity using the Uclust algorithm in QIIME (Version 1.9.1). A representative sequence for each OTU was selected and the taxonomic information was annotated using QIIME (Version 1.9.1) and the Green Gene database (Release 13_8_99). Richness estimates and diversity indices including Chao1, Observed OTUs, Good's coverage, phylogenetic diversity whole tree (PD whole tree), and Shannon's index were calculated using QIIME (Version 1.9.1). A principal coordinate analysis (PCoA) based on the weighted UniFrac distances was conducted to compare all samples.

Statistical analysis: To test the difference of ruminal fermentation parameters between the experimental treatments, multiple comparisons were performed using Duncan's method. For microbial community analysis, differential abundance phyla and genera Wilcoxon rank sum test was used, and P values corrected for multiple testing with the help of Benjamin & Hochberg method by R software (Version 167 3.3.1). GraphPad Prism 7.0 software was used to perform statistical analysis on the data for microbial relative abundance and alpha and beta diversity indices.

RESULTS

Ruminal Fermentation parameters: Results of ruminal fermentation parameters are presented in Table 2. This study results showed that ruminal fermentation parameters were not influenced by experimental treatment (P>0.05).

Ruminal bacteria composition at Phylum: Results of experimental treatment on ruminal phyla level are presented in Fig. 1A. Within these phyla, *Planctomycetes* were observed to be the least, while the most abundant phylum among the observed 11 phyla was *Bacteroidetes.* The abundance of *Bacteroidetes* was 59.82 %. The second most abundant phyla among was *Firmicutes* with an average abundance of 29.75%. as presented in Figure 1A, among the all 11 detected phyla these two phyla accounted for the majority of the microbiota. In both experimental treatments, *Bacteroidetes*, *Firmicutes* and *Synergistetes* had significant differences in ruminal bacteria community composition and relative abundance (P<0.05, Fig1B-D,).

Ruminal bacteria composition at Genus: The results of ruminal bacteria composition at genus level are presented in Figure 2A. The results of microbiota composition down to the genus level represents that there were 27 taxa (Figure 2A). Among all of 27 observed taxa, the most abundant taxa were *Prevotella* (35.04%) and *Ruminococcus* (7.52%). However, 37.68% of the sequences were unclassified at the genus level. Figure 2A represents the relative abundance of genera in ruminal samples, and highlights that *Succiniclasticum*, *Moryella*, and *Comamonas* were significantly different between the two experimental groups (P < 0.05).

Ruminal microbial colonies in different diet groups: This study showed that diversity and richness in the ruminal microbial composition differed significantly between CON and SWE groups at Chao 1 (P<0.05, Fig. 3B). The observed species, simpson index and Shannon index were not significant in the SWE experimental treatment as compared to the CON experimental group (P>0.05, Fig. 3A, 3C-D). The β-diversity was further analyzed as shown in the PCoA (Principal Coordinate Analysis) in Figure 3E-F. In the current experiment, PCoA plots that were based on the Bray-Curtis distance matrix represents that there was a significant separation between CON and SWE groups using PC2 (P<0.01, 16.66%, Fig. 3E). However, PCoA plots that were based on the Bray-Curtis distance matrix also represents that there was no significant separation between CON and SWE groups using PC1 (P>0.05, 45.2%, Fig. 3F).

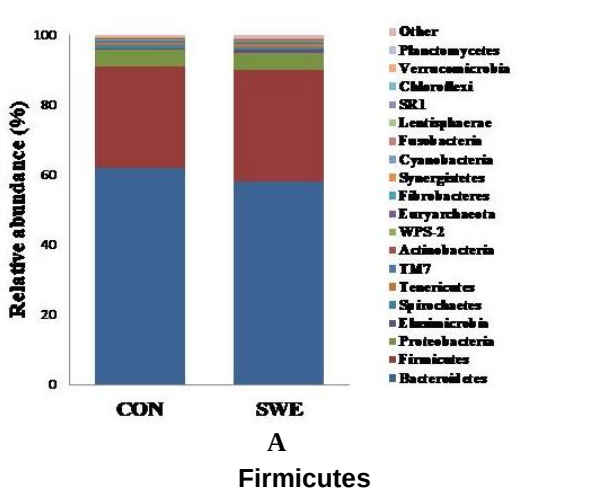
LEfSe analysis of rumen microbiota: The cladogram based on LefSe analysis showed that 2 and 5 biomarker genera were identified in the CON and SWE cohorts (Fig. 4A). Furthermore, as compared to the CON experimental treatment, the SWE group had *Flavobacteriia*, *Synergistia*, *Sphingobacteriales* and *Betaproteobacteria* that were increased significantly in relative abundanc (P<0.05, Fig. 4B), and 4C0d-2 and *Bacteroidia* were increased significantly CON diet as compared to the animlas in the SWE (P<0.05, Fig. 4B).

Predicted Functions of Bacteria Attached to Dietary SWE Treatments: Fig. 5 explored the functional predictions of all sequencing data via PICRUSt2. Results of functional predictions representes that microbial communities were mainly involved in metabolism (50.28%) (Fig. 5A), that were Carbohydrate Metabolism (10.65%) and Amino Acid Metabolism (10.55%) (Fig. 5B). In Fig. 5C it has been seen that there were three pathways that further explored the level 5 KEGG Orthology corresponding to their top 10 abundances. LefSe (least discriminant analysis value LDA of 4) analysis showed that biotin metabolism was upregulated in the SWE-supplemented group.The CON group was mainly involved in pathways related to Arginine and proline metabolism, Glycosphingolipid biosynthesis - globo series, Polyketide sugar unit biosynthesis, and Energy metabolism (p < 0.05, Fig. 5D).

DISCUSSION

Numerous studies have demonstrated that plant and animal by-product derived bioactive compounds are not only able to enhance the animal's ability to resist or diminish diseases by regulating the abundance of its gut microbiota but also enhance production performance by supporting gut microbiota, nutrient digestibility and immune system (Salsabil *et al.*, 2022; Orinetha *et al.*, 2022; Saad *et al.*, 2023). In the current study, *Laminaria japonica* powder was used in the diet of animals to evaluate its impact on rumen microbiota. When evaluated *in vitro* using rumen batch cultures, *Laminaria japonica* powder showed no impact on total VFA production, pH, or individual VFAs (Wang *et al.*, 2009), which was in agreement with the finding of this study. In the current study similar finding has been observed in vivo when *Laminaria japonica* powder was added in the diet of bulls it has not effect on the ruminal fermentation parameters including individual or total VFAs production. In contrast, in another study, the significant increase in rumen TVFA, acetate and propionate after addition of 5% (DM basis) seaweed meal in the diet has been observed (Xue *et al*, 2019). These findings suggest that at higher levels of seaweed meal promote rumen carbohydrate metabolism, which is consistent with the trend of the results of the present study. Thus, variation trends in VFAs production observed in the current study may help exploring the ecology of the ruminal microbiota along with mechanism how *Laminaria japonica* powder influence ruminal fermentation pattern *in vivo*.

The results of rumen flora analysis at the portal level showed that inclusion of *Laminaria japonica* powder in the diet of bulls reduce the level of *Bacteroidetes* populations.



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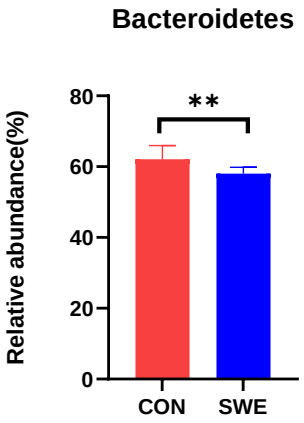
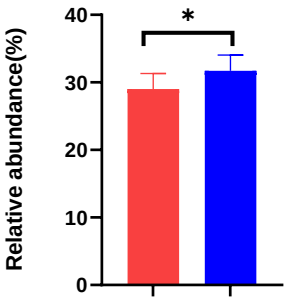


B

Fig. 1: (**a**) Relative abundance of microbiota at the phylum level. (**b**) Ratio of *Bacteroidetes* in the two groups. (**c**) Ratio of *Firmicutes* in the two groups. (**d**) Ratio of *Synergistetes* in the two groups. n = 10, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significance.



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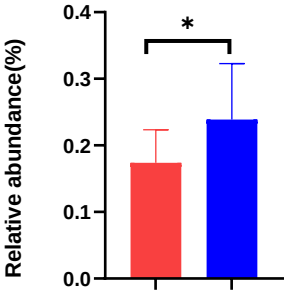

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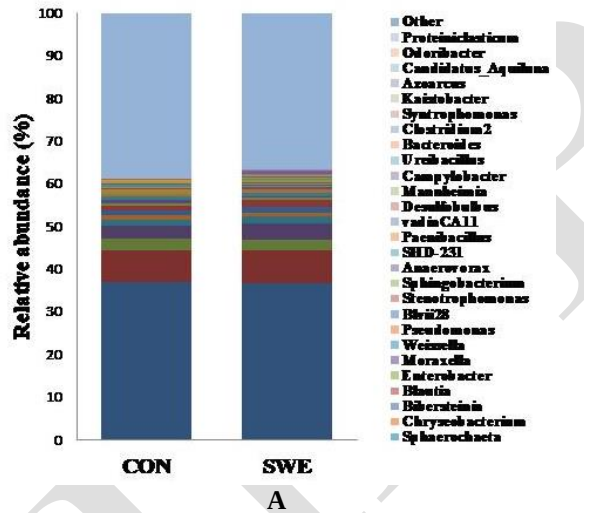

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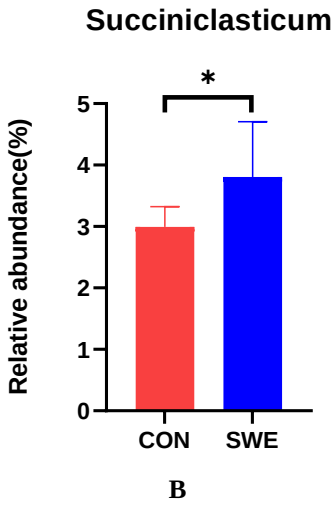
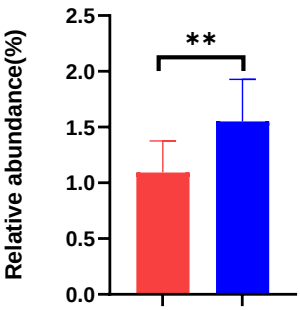


B

Fig. 2: (**a**) Relative abundance of microbiota at the genus level. (**b**) Ratio of *Succiniclasticum* in the two groups. (**c**) Ratio of *Moryella* in the two groups. (**d**) Ratio of *Comamonas* in the two groups. n = 10, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significance.



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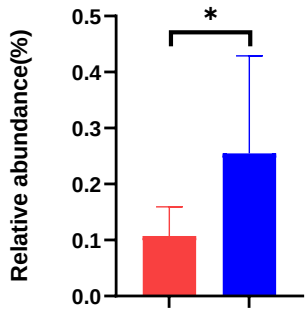

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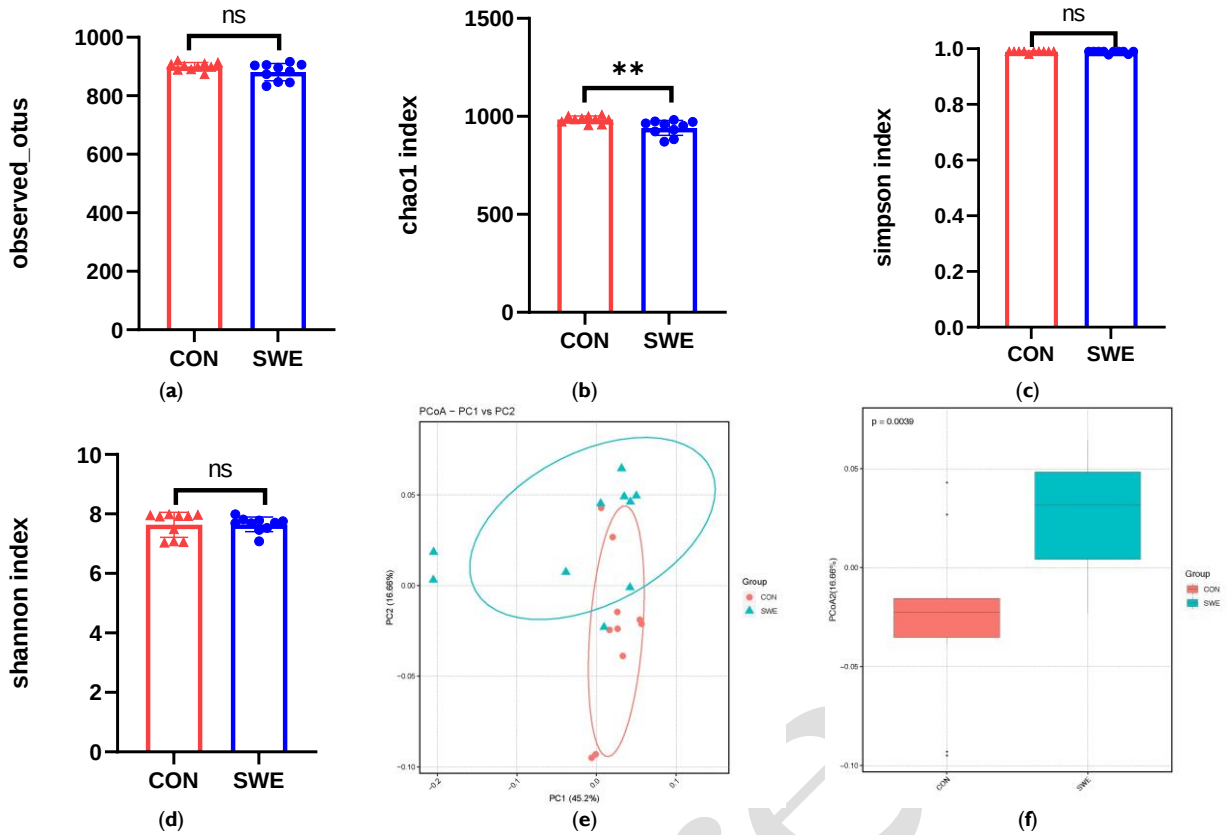

Fig. 3: Analysis of the diversity of rumen microflora in different dietary groups. (**a-d**) Alpha diversity (observed_otus, Chao1index, Shannon index, Simpson index);(**e**) beta diversity indicated by microbial community weighted unifrac (PCoA) plots. (**f**) PCoA plot of the Bray-Curtis distance matrix. n = 10, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significance.

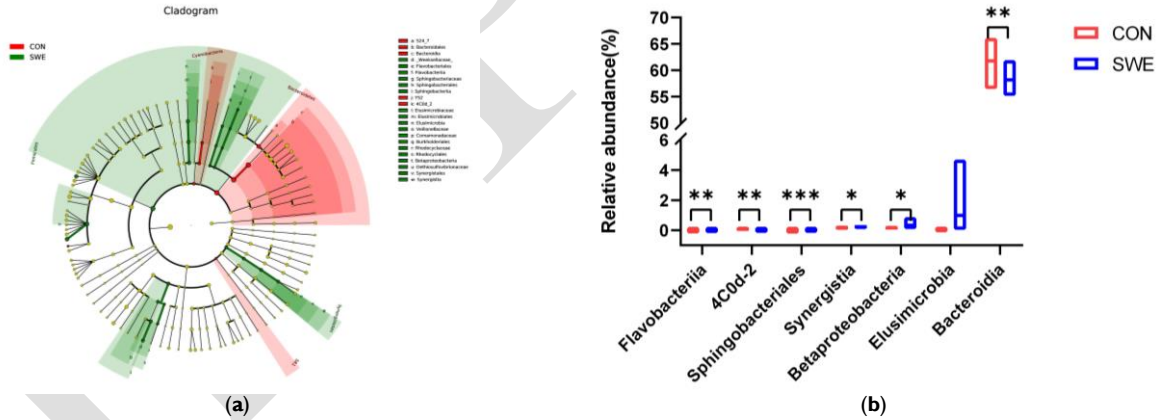

Fig. 4: Effect of feeding different diets on the relative abundance of microbiota at the genus level. (**a**) Cladogram analysis of microbiota in CON vs SWE;(**b**) Relative abundance of microbiota in the two groups. n = 10, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns indicates no significance

The findings of reduced level of *Bacteroidetes* populations in the bulls fed *Laminaria japonica* powder contradicted the findings of previous *in vitro* studies, where the inclusion of *Laminaria japonica* powder resulted in similar bacterial population (Bach *et al.*, 2008). These findings are also contradicted the findings of another study where bacterial population were even larger as compared to control (Wang *et al.*, 2009). The reduction of bacterial population in the current study could be attributed with antimicrobial population of *Laminaria japonica* powder because it has been reported that *Laminaria japonica* contains various antimicrobial contents like fucoidans, laminarin, and pure phlorotannin (Perez *et al.*, 2016), therefore, in the current study it could be assumed that lower *Bacteroidetes* populations in rumen was due to *Laminaria japonica* powder antimicrobial properties. In contrast, feeding kelp powder significantly enhanced the *Firmicutes* populations, supporting previous *in vitro* studies where the inclusion of kelp powder resulted in bacterial population sizes larger than those of control samples. It has been shown that kelp powder has a prebiotic effect, probably due to the addition of kelp powder to

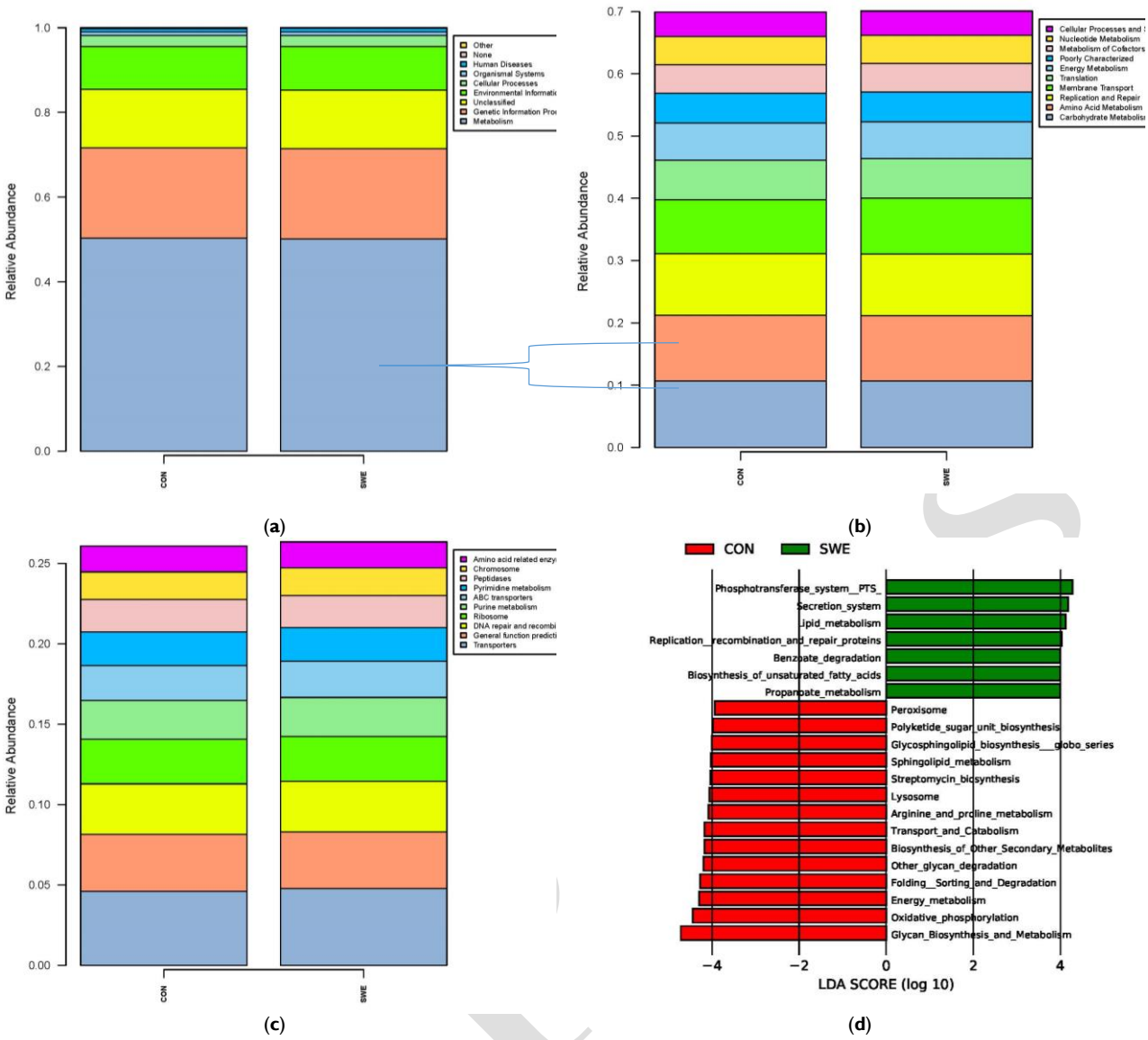

Fig. 5: Influence of feeding different diets on the function of intestinal flora. n = 10.

Table 2: Effects of kelp powder on rumen fermentation of finishing bulls

Items	Dietary treatment		SEM[3]	*P*-value
	CON[1]	SWE[2]		
pH	6.68	6.70	0.006	0.88
NH3-N mM/L	8.31	8.53	0.783	0.84
TVFAd (mM)	88.52	98	7.776	0.41
Acetate （mM)	49.44	53.79	3.788	0.44
Propionate （mM)	20.74	25.18	2.999	0.32
Isobutyrate （mM)	0.65	0.82	0.057	0.05
Butyrate （mM)	16.16	16.46	1.431	0.89
Isovalerate （mM)	0.91	1.03	0.106	0.45
Valerate （mM)	0.61	0.73	0.099	0.42
Acetate (%)	55.86	55.51	1.168	0.83
Propionate(%)	23.44	25.07	1.349	0.41
Isobutyrate(%)	0.73	0.87	0.058	0.11
Butyrate(%)	18.25	16.78	0.579	0.10
Isovalerate(%)	1.03	1.06	0.067	0.72
Valerate(%)	0.7	0.73	0.058	0.73
Acetate/ Propionate	2.39	2.29	0.136	0.62

[1] control group.[2] kelp powder group.[3] standard error of the mean.

improve the fermentation efficiency of the *Firmicutes* populations of the SWE group (Zhou *et al.*, 2018). In the current study, consistent VFA production was seen both in control and experimental diet that could be attributed improve fermentation efficiency of the *Firmicutes* populations of the SWE group. A previous study reported that both VFA molar portions and microbial relative abundance were influenced by seaweed feeding in ruminants (Zhou *et al.*, 2018). In the current study, lower relative abundance of the butyrate-producing bacteria *Firmicutes* was observed in the animals fed *Laminaria japonica* that resulted in reduce Butyrate (%) production in bulls fed *Laminaria japonica*.

The results of ruminal microbial population at the genus level showed that the relative abundance of *Succiniclasticum*, *Moryella*, and *Comamonas* was higher in animals raised on *Laminaria japonica* feeding as compare to control group. *Succiniclasticum* is one of the dominant groups of rumen microbial communities and a product of cellulose catabolism by Prevotella, and is capable of being converted to propionate, which is the the most important precursor of glucose in ruminants and produces an important role in the rumen fermentation process (Van G, 1995; Taiwo *et al.*, 2024). *Moryella* is able to catabolize carbohydrates and produce VFA, providing a source of energy and improving body health (Cheng *et al.*, 2022; Shen *et al.*, 2023). Previous studies have shown that

Comamonas is capable of producing butyrate (Pryde *et al.*, 2002), which has a reparative effect on the rumen epithelial barrier (Carlier *et al.*, 2007). It may be due to the fact that feeds containing kelp meal contain more fermentable carbohydrates, which further accumulate higher amounts of glucose, which generates pyruvate through glycolysis, and the accumulation of pyruvate is further converted to propionate, that resulted in an increase in the three genera *Succiniclasticum*, *Moryella*, and *Comamonas* (Pereira *et al.*, 2015; Pitta *et al.*, 2016). The result of relative abundance of these three genera suggests a beneficial effect on ruminant body health as the addition of seaweed meal facilitates rumen digestion, absorption and utilization of nutrients.

The LEfSe analysis of variance showed a significant increase in *Flavobacteriia*, *Synergistia*, *Sphingobacteriales* and *Betaproteobacteria* in the SWE group, whereas both *Flavobacteriia* and *Sphingobacteriales* belong to the thick-walled phylum, which has been shown to promote energy uptake and fiber decomposition in mice (Kameyama and Itoii, 2014; Li *et al.*, 2024), while Clemmons (Clemmons *et al.*, 2019) showed that *Flavobacteria* play a povital role in regulating effeciecy of feed and are able to increase the nutrient utilization, enabling the addition of kelp powder to provide energy and maintain rumen health in finishing bulls.

In the current study, *Laminaria japonica* powder feeding influenced the predicted metabolic pathways in bulls and altered predicted metabolic pathways suggested that *Laminaria japonica* powder have potential to promote microbial fermentation by enhancing upregulation of metabolic pathways. It has been reported that concentration of fatty acid is higher in seaweeds (Van Ginneken *et al.*, 2011). The enriched "Biosynthesis of unsaturated fatty acids" and "Glycosphingolipid biosynthesis" pathways observed in kelp powder-fed bulls indicate that in diets low in fatty acids, the ruminal microbiome could have an excellent capacity to metabolize fatty acids when *Laminaria japonica* powder is added in the diet of bulls. Similar results also have been reported in a recent study (Zhou *et al.*, 2018). In the current study, higher "butanoate metabolism" was observed in the animals that were raised on *Laminaria japonica* powder diet and higher "butanoate metabolism" level in the current study represents rumen butyrate metabolizing microbiome were active in these animals. It could be supposed that rumen butyrate metabolizing microbiome could contribute to the reduced butyrate concentration in the rumen. It has been reported that *Laminaria japonica* contained aromatics, phenols, alkanes and alkenes, other oxygen-containing compounds, and some nitrogen-containing compounds (Li *et al.*, 2013). Predicted function revealed enriched 'Benzoate degradation pathways observed in kelp powder-fed bulls. It is well known that benzoate is compound that work as an essential mediator in the aerobic and anaerobic metabolism of aromatic compounds (Prajapati *et al.*, 2016). Enriched 'Benzoate degradation pathways observed in animals reared on kelp powder-feed represents that animals reared on kelp powder-feed have strong ability to metabolize dietary aromatic compounds. Phlorotannins are a type of tannins found in brown algae such as kelps (Wang *et al.*, 2009) and it has been reported that tannins affect arginine and proline metabolism (Yao *et al.*, 2022). In the current study, the "arginine and proline metabolism" pathway was negatively influenced in kelp powder-fed bulls indicating that tannin content have adverse effects even at a low-level feeding. In the current study, only ruminal microbiome was investigated along with ruminal metabolites parameters and ruminal fermentation parameters. It is well known that ruminal microbiome is directly related with animal health and production parameters. Therefore, study of kelp powder to ruminant for longer time and its impact on animal health and production traits is required.

Conclusions: Drawing upon 16S rRNA high-throughput sequencing analysis of rumen microbiota, this investigation provided a comprehensive overview of bacterial populations in the rumen and their proportional representation in response to kelp powder-supplemented diets. The findings indicated that incorporating kelp powder (0.7%, DM basis) did not adversely impact ruminal fermentation characteristics or the composition of rumen bacterial communities. The current study used a very low level of kelp powder in the finishing bull's diet, thus, a higher level of kelp powder in the finishing bull's diet and its impact on ruminal bacteria composition and animal products like meat and carcass are warranted.

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