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RESEARCH ARTICLE

Physiological Variation in Ruminal Microbiota under Altered Energy Levels in Starter Ration of Suckling Angus Calves

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ABSTRACT

The present study aim was to see the effects of different starter rations energy levels in the diet on ruminal microbial diversity, bacterial community structure and microbial diversity indices of rumen bacterial communities in Angus calves. For this, 18 suckling Angus calves were distributed into 3 different treatment groups. Dietary treatments were: Low metabolizable energy ration (LME); Medium metabolizable energy ration (MME) and High metabolizable energy ration (HME). Results of bacterial community revealed that most abundant phylum was *Firmicute.* At phyla level, *TM7* and *Fibrobacteres* showed significant higher relative abundance in LME group than HME group. Under different dietary energy, 59 genera showed significant correlation with physiological production parameters. These results provide new information for the understanding of the dietary energy level effect on ruminal microbial community interactions and are of great importance for optimizing energy supply strategy for suckling calves.

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INTRODUCTION

Microorganisms of rumen degrade feed stuff of mature ruminants to provide nutrients including essential amino acids, vitamins as well as volatile fatty acids (VFAs) (Henderson *et al*., 2015). The diversity of rumen microbiota varies during different stages of life (Qiu *et al*., 2019). It has been reported that ruminants during early stage of life, especially from 0-21 days, are considered as monogastric. The reason behind considering ruminants as monogastric during early stage of life is underdevelopment of rumen both physiologically and anatomically (Dias *et al*., 2017). At pre-ruminant stage of life, feed of ruminants is primarily milk, which bypasses the main rumen segment and enters directly into abomasum via esophageal groove (Davis and Drackley, 1998). The start of solid feed intake around 21 days of age starts the process of development of rumen of ruminants with the help of rumen microbiota. It has been reported that rumen microbial community formation and activity has significant impact on rumen development (Lv *et al*., 2019). However, ruminal microorganism formation and activity mainly depends upon the type of feed of pre-ruminates (Jiang *et al*., 2019; Lv *et al*., 2019).

In a study of pre-ruminants, it has been observed that only diet of milk, compared with solid feed plus milk, influences the microbial communities of gastrointestinal tract and feces differently (Guzman *et al*., 2016). It has also been reported that a diet containing higher amount of fermentable carbohydrates caused a huge variation in bacterial population (Liu *et al*., 2015). Similarly, other researchers also reported that in dairy animals, rumen microbial population varied greatly not only in terms of bacterial species but also in number and composition between diet containing highly fermentable carbohydrates *vs* control diet (Mao *et al*., 2013). Therefore, based on previous studies review, it is concluded that different feeding regime influences diversity in ruminal bacterial population (Liu *et al*., 2015).

In the developing rumen, the colonization process of microorganism in rumen is key factor for better rumen functions that can affect efficiency and stability of digestion at mature stage of life (Heinrichs, 2005; Yáñez-Ruiz *et al*., 2015). In modern dairy production

management, newborn calves are separated from the dam after birth and reared on either milk replacer or whole milk. Scientists have explored effect of dam nutrition, feeding materials on rumen ecology, rumen fermentation and production from very soon after birth into adulthood in dairy calve. However, in meat production system, where the offspring remains with the dam until weaning, a little research has been done to evaluate the effect of dam nutrition, feeding materials on rumen ecology, and rumen fermentation from very soon after birth. In this backdrop, the current project evaluated the alterations in rumen fermentation processes and ruminal microbial community following variation in energy levels in starter ration of sucking Angus calves.

MATERIALS AND METHODS

Ethics Statement: Animal Welfare and Use Committee of Hunan Agricultural University approved the experimental procedure of current study.

Diet composition and animal management: In this study, eighteen Angus calves of about twenty days old with initial BW = 43.4±4.25 kg were selected. Animals were divided into 3 different experimental groups each comprising of three replicates with two calves in each replicate. Dietary treatments were: Low metabolizable energy (LME) that was 11.19 MJ/Kg; Medium metabolizable energy (MME) that was 12.38 MJ/kg and High metabolizable energy (HME) that was 13.62 MJ/kg. The composition of pelleted starter rations is shown in Table 1. Besides, pelleted starter ration, calves were offered whole oat hay ad-libitum. Milk was provided to each calf from their dams by suckling method at 07:00 to 18:00 every day.

Samples collection: Newly fed and refused starter was recorded daily to calculate individual starter intake. Ruminal samples collection procedure is described previously (Chen *et al*., 2020). Three aliquots each containing sample 1 mL were placed in liquid nitrogen for 16S rRNA analysis.

DNA extraction and pyrosequencing: DNA extraction and 16S rRNA pyrosequencing procedure has been described previously (Chen *et al.,* 2020). Briefly, clear supernatant was collected following centrifugation of rumen fluid sample (1.5 mL). DNA was extracted using DNA isolation kit (Magen, China) and 16S rRNA genes from V3-V4 region were amplified with the help of specific primers. PCR reactions were performed in triplicate and the PCR products were purified using agarose gel electrophoresis.

Data analyses: QIIME (Version 1.9.1) was used to remove low quality sequences of raw reads (Caporaso *et al*., 2010). The filtered raw reads tags were merged using FLASH, Version 1.2.7 (Magoč and Salzberg, 2011). The chimeric tags were removed using Usearch software (Version 8.1.1861) (Edgar *et al*., 2011). Operational taxonomic units (OTUs) (97% similarity) were made from the selected tags (DeSantis *et al*., 2006).

Statistical analysis: R software (Version 3.3.3) was used for the data analysis. Duncan's multiple rang test was used to analyze differences among groups. Wilcoxon rank sum test was used for differential abundance of genera. P value<0.05 was considered significant. Correlations between microbial proportion and physiological / production parameters (including dry matter intake, growth index, nutrient digestibility, ruminal fermentation parameters) were determined using R software (Version 3.3.3, pheatmap package).

RESULTS

Bacterial Population Comparison Phyla and Genera Levels: Raw tags (81,441), with an average length of 407 bp, were allotted, based on a 97% similarity cut-off, to 68,243 operational taxonomic units (OTUs). Bacterial population comprising of top 10 phyla and genera levels are presented in Fig. 1. Eight (08) phyla (relative abundance ≥0.1%) was observed across all the experimental diets (Table 2). *Fibrobacteres* was found to be the minimum while *Firmicutes* was the most abundant phylum (54.16%), followed by *Bacteroidetes* (35.78%). *TM7* and *Fibrobacteres* was observed to be at significantly higher relative abundance in LME group than in HME group (Table 2). At genera level, 20 taxas shared genera with a relative abundance (≥0.1%) were found (Table 3). The most abundant taxa was *Prevotella* (17.90%) followed by *Ruminococcus* (5.23%), *Succiniclasticum* (1.56%), *CF231* (0.89%) and *Sharpea* (0.69%). The top 10 genera, based on relative abundance, are presented in Fig. 1B. No significant difference was observed at genera level among different diet groups (Table 3) except *Atopobium*, *Wautersiella* and *Actinobacillus*.

Growth performance, rumen fermentation parameters and nutrient digestibility in calves: It was found that the rumen fermentation indices were related to the ruminal bacteria community (Fig. 2). In detail, *Haemophilus*, *Mitsuokella*, *Acinetobacter*, and *Erwinia* (P<0.05) were positively correlated with valerate concentration in rumen while, *Porphyromonas*, and *Vibrio* (P<0.05) were negatively correlated with valerate concentration in rumen. *SHD-231*, *Mycoplasma*, *Shuttleworthia*, *Mycetocola*, *Coprococcus* were positively correlated with butyrate concentration. However, butyrate concentration was found to be correlated negatively with *Halomonas*. *GW-34* was positively correlated with acetate concentration in the rumen while *Faecalibacterium*, *Phormidium*, *Suttonella*, and *Collinsella* were negatively correlated with acetate concentration in the rumen. *Faecalibacterium*, *Treponema*, *Collinsella*, *Schwartzia*, and *Halomonas* were negatively correlated with TVFA concentration. *Schwartzia*, *Halomonas*, and *Yonghaparkia* were negatively correlated with propionate concentration. *Christensenella*, and *Lachnobacterium* were positively correlated with propionate concentration. *Anaeroplasma*, *Pseudoramibacter_Eubacterium* and *Pseudoalteromonas* were positively correlated with organic matter digestibility. *Coprococcus* and *Luteimonas* were negatively correlated with organic matter digestibility. *Anaeroplasma*, *Pseudoramibacter_Eubacterium*, *Pseudoalteromonas* and *vadinCA11* were correlated positively with dry matter digestibility. *Solibacillus* and *Luteimonas* were negatively

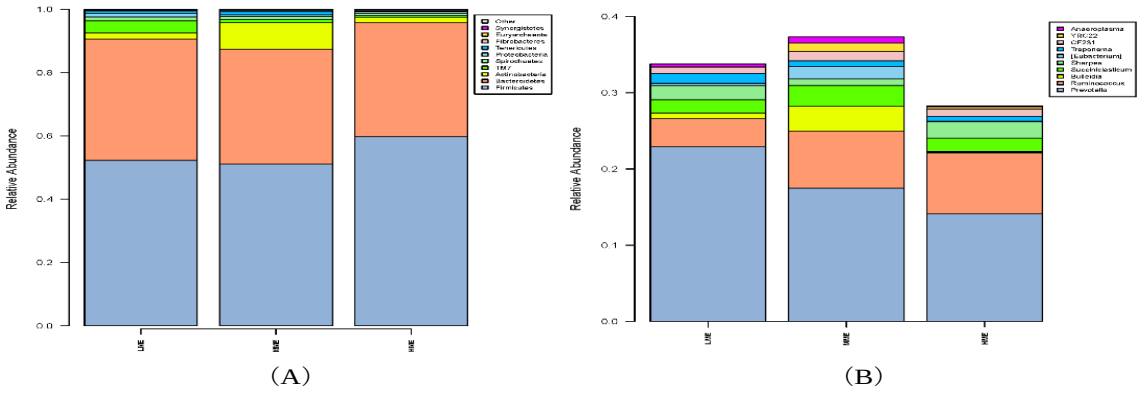

Fig. 1: Bacterial community structure variation in different groups. The relative abundance of the top 10 most abundant (A) phyla and (B) genera is shown. Each bar represents the relative abundance of each sample. Each colour represents a particular phylum or genus.

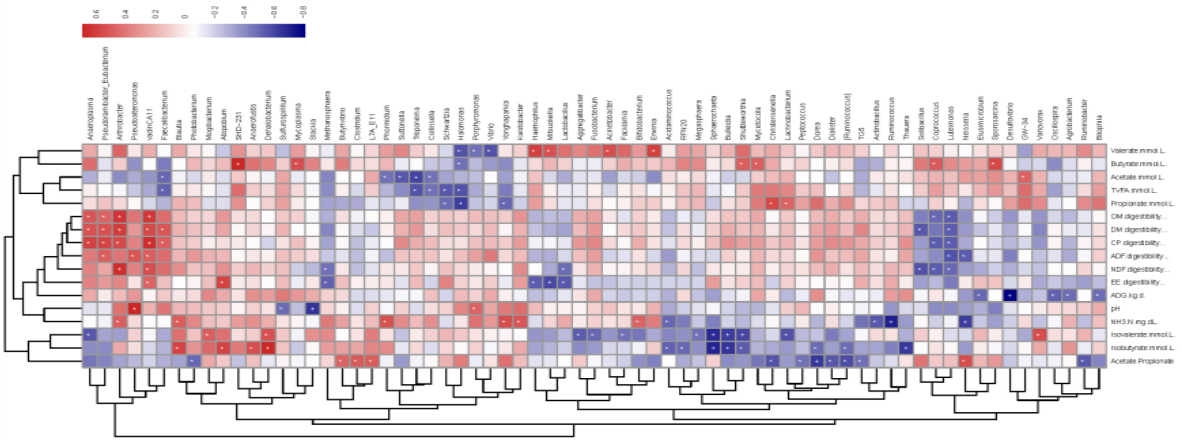

Fig. 2: Correlation between physiological / production parameter and genus abundance. The down and left hierarchical cluster based on the corresponding correlation matrix between physiological / production parameters and genus abundance.

Table 1: Composition and nutrient levels of content rate diet (DM basis) %

Items	LME[3]	MME[4]	HME[5]
Ingredients			
Corn	5.00	30.60	46.50
Soybean meal	16.00	17.00	18.00
Wheat bran	59.00	29.90	9.00
Soybean protein concentrate	3.00	3.50	6.00
Cotton seed meal	7.00	8.00	7.00
Soya-bean oil	0.00	1.00	3.50
Premix [1)]	5.00	5.00	5.00
Molasses	5.00	5.00	5.00
Total	100	100	100
Nutrient levels [2)]			
DM	87.00	88.00	89.00
CP	23.17	20.77	23.02
EE	2.68	2.73	3.47
NDF	22.11	18.10	11.21
ADF	10.40	8.41	7.35
ME[2](MJ/kg)	11.19	12.38	13.62

[1]One kilogram premix provided the following: VA 260 00 KIU, VD3 100 00 KIU, VE 70 KIU, Fe 8000 mg, Mn 6290 mg, Zn 816.3 mg, Cu 1200 mg, I 1200 mg, Se 600 mg, Co 200mg, Ca 150 g, P 30 g。 : [2]ME was a calculated value and others were measured values: [3]LME=low metabolizable energy calf starter, the same as below: [4]MME=Medium metabolizable energy calf starter, the same as below: [5]HME=High metabolizable energy calf starter, the same as below.

correlated with dry matter digestibility. *Anaeroplasma*, *Pseudoramibacter_Eubacterium*, *Pseudoalteromonas* and *vadinCA11* were positively correlated with crude protein digestibility. *Coprococcus* and *Luteimonas* were negatively correlated with crude protein digestibility. *Pseudoramibacter_Eubacterium*, *Pseudoalteromonas*, and *vadinCA11* were positively correlated with acid detergent fiber digestibility. *Luteimonas* and *Neisseria* were negatively correlated with acid detergent fiber digestibility. *Arthrobacter*, and *vadinCA11* were correlated positively with neutral detergent fiber digestibility. *Methanosphaera*, *Lactobacillus*, *Solibacillus*, *Coprococcus*, *Luteimonas* were correlated negatively with neutral detergent fiber digestibility. *Faecalibacterium* and *Atopobium* were positively correlated with ether extract digestibility. *Methanosphaera*, *Haemophilus*, *Mitsuokella*, *Lactobacillus* were negatively correlated with ether extract digestibility. *Elusimicrobium*, *Desulfovibrio*, *Oscillospira*, *Agrobacterium*, and *Bilophila* were negatively correlated with average daily gain. *Pseudoalteromonas* and *Porphyromonas* were positively correlated with pH while Sulfurospirillum and Slackia were negatively correlated with rumen pH. Arthrobacter, *Blautia*, *Phormidium*, *Yonghaparkia*, *Kaistobacter* and *Bifidobacterium* were positively correlated with NH_3-N. *Acidaminococcus*, *Actinobacillus*, *Ruminococcus* and *Neisseria* were correlated negatively with NH_3-N. *Mogibacterium*, *Variovorax* and *Dehalobacterium* were positively correlated with isovalerate. *Anaeroplasma*, *Aggregatibacter*, *Fusobacterium*, *Facklamia*, *Megasphaera*, *Sphaerochaeta*, *Bulleidia*, *Shuttleworthia*, *Lachnobacterium* were negatively correlated with isovalerate. *Blautia*, *Atopobium*, *Anaerofustis* and *Dehalobacterium* were positively correlated with isobutyrate concentration in the rumen. *Acidaminococcus*, *RFN20*, *Sphaerochaeta*, *Bulleidia*, *Shuttleworthia*, *Dorea*,

Table 2: Phylum-level composition (relative abundance ≥0.1%) of the rumen among different groups. Values are Median. a,b Values in the same row with different superscripts differ significantly (P<0.05)

Phylum	Relative abundance (%)				SEM	P
	All	LME	MME	HME		
Firmicutes	54.16	49.78	52.10	62.30	1.110	0.119
Bacteroidetes	35.78	38.48	36.66	33.23	0.444	0.927
Actinobacteria	1.49	1.53	2.03	1.49	0.051	0.983
TM7	0.92	1.44[a]	0.77	0.53[b]	0.079	0.018
Spirochaetes	0.84	1.15	0.76	0.79	0.036	0.236
Tenericutes	0.53	0.62	0.84[a]	0.27[b]	0.048	0.050
Proteobacteria	0.56	0.94	0.65	0.40	0.045	0.082
Fibrobacteres	0.11	0.14[a]	0.17	0.02[b]	0.013	0.057

Table 3: Shared genera with a relative abundance ≥0.1% in all samples among different groups. Values are mean. a,b Values in the same row with different superscripts differ significantly (P<0.05)

Phylum	Genus	Relative abundance (%)				SEM	P
		All	LME	MME	HME		
Bacteroidetes	*Prevotella*	17.90	20.51	19.09	6.40	0.717	0.244
	YRC22	0.08	0.05	0.14	0.13	0.009	0.144
	CF231	0.89	0.77	1.44	0.91	0.059	0.692
Firmicutes	*Ruminococcus*	5.23	2.68	8.21	5.96	0.463	0.095
	Bulleidia	0.44	0.41	1.65[a]	0.07[b]	0.138	0.010
	Sharpea	0.69	0.35	0.57	0.78	0.036	0.414
	Succiniclasticum	1.56	1.83	1.77	1.38	0.040	0.920
	[Eubacterium]	0.11	0.15	0.51[a]	0.02[b]	0.042	0.022
	Solibacillus	0.17	0.10	0.34	0.19	0.020	0.778
	Butyrivibrio	0.31	0.43	0.23	0.23	0.019	0.476
	Coprococcus	0.22	0.17	0.28	0.40	0.020	0.484
	Clostridium	0.21	0.22	0.11	0.19	0.009	0.422
	RFN20	0.12	0.06	0.19	0.12	0.011	0.244
	Oscillospira	0.15	0.13	0.15	0.15	0.002	0.834
Spirochaetes	*Treponema*	0.82	1.12	0.74	0.79	0.035	0.209
Fibrobacteres	*Fibrobacter*	0.11	0.14[a]	0.17	0.02[b]	0.013	0.057
Tenericutes	*Anaeroplasma*	0.33	0.32	0.48[a]	0.14[b]	0.028	0.019
Proteobacteria	*Suttonella*	0.21	0.52[a]	0.22[a]	0.05[b]	0.040	0.008
	Desulfovibrio	0.17	0.14	0.1	0.19	0.004	0.675
Actinobacteria	*Bifidobacterium*	0.09	0.17	0.13[a]	0.03[b]	0.012	0.012

Ruminococcus and *hauera* were negatively correlated with isobutyrate concentration in the rumen. *Butyrivibrio*, *Clostridium*, *L7A_E11* and *Neisseria* were positively correlated with acetate to propionate ratio. *Photobacterium*, *Christensenella*, *Peptococcus*, *Dorea*, *Dialister*, [*Ruminococcus*], *TG5* and *Ruminobacter* were negatively correlated with acetate to propionate ratio.

DISCUSSION

Higher energy contents in the diet of weaning calves are often desired for increased growth (Rauba *et al*., 2019). The increase in energy contents accomplished by additional milk or by increase energy level and density of milk or starter ration (McLeod and Baldwin, 2000). Generally, it is considered that increasing the energy level in the diet of weaning calves did not alter the dry matter and organic matter intake but improve feed efficiency.

In this study, we studied the effects of different starter rations energy levels in the diet on bacterial community structure and correlation between production parameters and genera abundance in Angus calves. We found that rumen samples from claves on low metabolizable energy and medium metabolizable energy starter ration showed higher ruminal microbiota diversity as compared to high metabolizable energy starter ration, which is consistent with previous literature showing decrease in microbial diversity following grain-based high fermentable carbohydrates diet (Wang *et al*., 2009; Kim *et al*., 2016).

About 90% of the bacterial population comprised of *Firmicutes* (54.16%) and *Bacteroidetes* (35.78%). Lv *et al.* (2020) also reported energy level of rations significantly increased the abundance of *Firmicutes* and *Bacteroidetes*. Our results showed that the calves fed on starter ration with low ruminal pH demonstrated higher abundance of *Firmicutes* in line with previous study (Kim *et al*., 2016). The digestion of complex carbohydrates is enhanced by *Bacteroidetes* which help in fermentation of organic matter (Jiang *et al.*, 2019). The numerical increase in *Bacteroidetes* abundance in the rumen of calves with low metabolizable energy diets could be explained by higher fiber contents in it due to higher wheat bran concentration in low metabolizable diet of calves. It has been reported that high fiber diet with relatively less metabolizable efficiency results in higher abundance of *cellulolytic* bacteria in lamb's rumen as well as in human's gut (Salonen *et al*., 2014). This may indicate the important role of *cellulolytic* bacteria in enhancing metabolic status of the low energy diets. The *cellulolytic* bacteria diversity can be explained by the composition of the low metabolizable energy diet that contains a high fiber due to higher concentration of wheat bran (Qian *et al*., 2018).

Fibrobacteres and *Fibrobacter* are commonly found in animals fed on fiber-rich diet and play important role in cellulose breakdown (Cui *et al.*, 2019). *Suttonella* genus belongs to the *Proteobacteria* plays a vital role in rumen metabolism although it is found in relatively low abundance. It has been commonly found in animals fed on diet containing higher starch contents (Kim *et al.*, 2016; Dias *et al.*, 2017; Cui *et al.*, 2019). We observed contrasting results showing high abundance of *Suttonella* in diet containing low metabolizable energy with lower level of

corn. Low metabolizable energy diet contained wheat bran in maximum quantity as compared to other diets, therefore, it is assumed that *Suttonella* species may also play some role in fiber degradation. Nevertheless, this assumption showed by verified by further large-scale studies. *Anaeroplasma* populations was found to be decreased at higher rate. This might be due to intolerance to decreased rumen pH (Loor *et al*., 2016). The lower abundance of *Tenericutes* in high metabolizable energy diet containing higher level of corn could be explained by lower rumen pH linked with higher concentration of grain in the diet. *Bifidobacterium*, a beneficial bacterium, enhances immunity and improves gut microenvironment by decreasing the levels of intestinal pathogens. In the current study, *Bifidobacterium* decreased as the metabolizable energy increased in the diet of calves by more addition of corn as compared to medium metabolizable energy diet which is contrary to the findings of Loor *et al.* (2016) who reported higher levels of *Bifidobacteria* at reduced rumen pH.

It has been reported that the abundances of *Fibrobacter*, *Treponema* became greater in fibrous feeds in ruminants (Lv *et al*., 2019). *Treponema*, saccharolytic bacteria, have ability to produce acetate and lactate. It has been observed that *Treponema* cannot use fiber, but it provides assistances to other bacteria to digest cellulosic materials (Lv *et al*., 2019). The abundance of *Treponema* in the rumen by utilizing fibrous feed indicates that *Treponema* have ability to reduce the total VFA concentration. The findings of current study that *Treponema* were negatively correlated with total VFAs concentration are in line with the findings of (Lv *et al*., 2019). Lower taxa *Clostridia* and *Clostridiales* have positive correlations with the acetate proportion and the A:P ratio (Lv *et al*., 2020) which is in agreement with the current experiment findings.

Conclusions: Our results show that the most abundant phylum was *Firmicute* among all the energy level ration groups. At phyla level, *TM7* and *Fibrobacteres* showed significantly higher relative abundance in LME group than HME group. Under different dietary energy, 59 genera showed significant correlation with physiological production parameters. These results provide new insights for the understanding of the dietary energy levels effect on ruminal microbial community interactions and are of great importance for optimizing energy supply strategy for suckling calves.

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Authors contribution: DC conceptualized the experiment. DC, FW, FL and MAUR handled experimental animals, collected samples, and analyzed the samples. HS and GZ carried out DNA extraction and 16S rRNA pyrosequencing and pyrosequencing data analyses. DC and MAUR wrote the manuscript.

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