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Liquid feeding regime and plant extracts affect rumen fermentation and bacterial community in calves

Zhang Rong

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE-GEMBLOUX AGRO-BIO TECH

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Zhang Rong

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Promoteurs: Yves Beckers

Diao Qiyu (CAAS, China)

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Abstract

Liquid feeding regime and nutrition of calves may produce long-term effect on growth, health, and milk production performance of the adults by influencing the establishment of ruminal microbiota. The objective of this thesis was to investigative the effect of early feeding regime and nutrition on rumen fermentation and bacterial community in calves, and then we follow calves for 6 or 9 months of age to detect any persistence of microbial programming effects.

In experiment 1, fifty-four Holstein calves were randomly allocated to one of three treatments and fed pasteurized waste milk (WM), whole milk (M), or milk replacer (MR) during 7 to 63 days of age. Male calves were slaughtered at age 2 months to measure the organ development of forestomach. Female calves were followed for 6 months to determine body weight, blood indices, rumen fermentation, and ruminal bacterial community. The results indicated that the average daily gain was lower but the concentration of total volatile fatty acids was higher in calves fed MR at 2 months of age. This may induce a microbial programming effect at 6 months of age that the ruminal propionate molar proportion was lower, but the ruminal pH and acetate/propionate ratio were higher, for the MR group. Calves fed WM had lower starter intake, rumen weight, but higher concentration of isovalerate at 2 months of age. Meanwhile, the concentrations of serum growth hormone, insulin-like growth factor 1, and the growth hormone to insulin ratio were higher, but the levels of insulin and nonesterified fatty acids were lower, for the WM group, at 2 months of age. Calves fed WM had a different ruminal bacterial composition at age 2 months. No difference was observed in growth, blood indices, rumen fermentation and microbiota between WM and M at 6 months of age.

In experiment 2, fifty-four female Holstein calves were randomly assigned to three treatments consisting of basal diet alone or supplemented with sanguinarine or resveratrol during 7 days to 6 months of age. Body weight was measured at the beginning of the

experiment and 2 or 6 months of age. Ruminal liquids were sampled at 1, 2, 3, 4, 5, 6, and 9 months of age to monitor rumen fermentation parameters, and samples at 3 and 6 months of age were used to analyze the bacterial community by high-throughput sequencing. The copy number of *Desulfovibrio* and methanogenic archaea were quantified using droplet digital PCR. Supplementing the basal diet with plant extracts affected rumen fermentation parameters as well as the bacterial community. In addition, the *Desulfovibrio* population increased after addition of sanguinarine or resveratrol, whereas the methanogenic archaea population decreased after addition of resveratrol. However, no difference were observed in rumen fermentation parameters and bacteria community structure among treatments at 6 months of age.

To sum up, liquid feeding regime and nutrition can affect rumen fermentation and bacterial community in calves. Calves fed WM had a different rumen fermentation and bacterial community at 2 months of age. However, this alteration can not persist to 6 months of age. Similarly, supplementation of sanguinarine or resveratrol to calves altered rumen fermentation parameters and microbiota during 3-5 months of age. But, no difference was detected at 6-9 months of age once the dietary treatment was ceased. It is noteworthy that calves fed MR had a higher concentration of TVFA in rumen, which might be a key factor that induced a more long-lasting effect on the rumen environment.

Key words: liquid feeding regime; plant extracts; rumen fermentation; bacterial community; calves

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Zhang Rong

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List of Abbreviations

ACE	Abundance-based coverage estimator
ADF	Acid detergent fiber
ADG	Average daily gain
ANOSIM	Analysis of similarity randomization test
A:P ratio	Acetate to propionate
BCVFA	Branch-chain volatile fatty acid
DGGE	Denatured gradient gel electrophoresis
ddPCR	Droplet digital PCR
EGF	Epidermal growth factor
GI tract	Gastrointestinal tract
GLM	General liner model
GH	Growth hormone
IGF-1	Insulin like growth factor-1
INS	Insulin
M	Milk
MR	Milk replacer
NDF	Netural detergent fiber
NEFA	Non-esterifier fatty acid
NH_3-N	Ammonia nitrogen
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
RES	Resveratrol
SAG	Sanguinarine
SARA	Subacute ruminal acidosis
SEM	Standard error of the mean
SI	Starter intake
SUN	Serum nitrogen
TGF	Transforming growth factor
TVFA	Total volatile fatty acid
VFA	Volatile fatty acid
WM	Waste milk

List of publications during PhD period

1. **Zhang, R.**, Zhang, W. B., Bi, Y. L., Tu, Y., Ma, T., Dong, L. F., Du, H. C., Diao, Q. Y. (2019). Sanguinarine and resveratrol affected rumen fermentation parameters and bacterial community in calves. ***Animal Feed Science and Technology,*** 251, 64-75.
2. **Zhang, R.**, Zhang, W. B., Bi, Y. L., Tu, Y., Beckers, Y., Du, H. C., Diao, Q. (2019). Y. Early feeding regime of waste milk, milk and milk replacer to calves has different effects on rumen fermentation and bacterial community. ***Animals***, 9, 443.
3. **Zhang, R.**, Diao, Q. Y., Zhou, Y., Yun, Q., Deng, K. D., Qi, D., Tu, Y. (2017). Decreasing the pH of milk replacer containing soy flour affects nutrient digestibility, digesta pH, and gastrointestinal development of preweaned calves. ***Journal of Dairy Science***, 100(1), 236-243.
4. **Zhang, R.**, Dong, X., Zhou, M., Tu, Y., Zhang, N., Deng, K., Diao, Q. (2016). Oral administration of Lactobacillus plantarum and Bacillus subtilis on rumen fermentation and the bacterial community in calves. ***Animal Science Journal***, 88(5), 755-762.
5. **Zhang, R.**, Zhou, M., Tu, Y., Zhang, N. F., Deng, K. D., Ma, T., Diao, Q. Y. (2015). Effect of oral administration of probiotics on growth performance, apparent nutrient digestibility and stress-related indicators in Holstein calves. ***Journal of Animal Physiology and Animal Nutrition***, 100(1), 33-38.
6. Diao, Q. Y., **Zhang, R. (co-first author)**, Tong, F. (2019). Review of strategies to promote rumen development in calves. ***Animals***, 9(8), 490.
7. Diao, Q. Y., **Zhang, R.**, Tu Y. (2017). Current research progresses on calf rearing and nutrition in China. ***Journal of Integrative Agriculture***, 16(12):60345-7.

1

General Introduction

1. Background

China produces a surprisingly large number of dairy cattle. As for 2018, China's dairy cow breeding stock numbered 10.38 millions, while its milk production reached 38.00 million tons. However, China is not on the list of developed cattle breeding countries. In China, the average milk yield per cow per year is about 6~7 tons, which is far below 8~10 tons in developed countries. Many problems exist in current rearing systems of calves and restrict the sustainable development of dairy cattle in China.

At the present, calf rearing in China adopts a traditional breeding system. The death rates of pre-weaned calves are high, which is due to many problems in the system of calf rearing. Firstly, the theoretical basis regarding calf physiology, rumen development, and the establishment process of rumen microorganisms is absent. Nutrient requirement of calf needs to be improved. Secondly, the feeding program is out of date. In China, dairy calves are commonly weaned at 10-12 weeks of age, whereas average weaning age is 8.4 weeks in the United States (USDA, 2002). Prolonged milk feeding increases labor and is less economical if an adequate starter intake for a less-stressful weaning could be attained earlier. Calves fed large amounts of liquid feed are less motivated to consume calf starter (Jasper and Weary, 2002; Quigley et al., 2006), and this decreases VFA production which is necessary to stimulate rumen development. Thirdly, the breeding objectives remain unclear. Calf raisers lack knowledge on the optimal growth rate for calves at different ages, and most of them know little about their own calves' growth rate. When poorly managed, calves often gain slowly, and thus the first mating age could be delayed from 14 months to 16-18 months. This seriously affects dairy herds' construction and turnover rate. By contrast, if calves gain too fast, it will increase their body fat deposition and impair their mammary gland development (Sejrsen and Purup, 1997). Fourthly, feeding facilities are out of date and do not match with the advanced feeding technology. Nowadays, large-scale dairy farms commonly wean calves early using milk replacer (MR). However, the supporting equipment and feeding systems remain relatively rare.

Calves are the reserve forces for dairy cattle. Scientific rearing strategy of calves is the basis of efficient cattle breeding. Early rearing of calves may produce long-term effect on the cattle. It is widely believed that the production potential of an adult cow is mainly determined by its genetic programming effects. However, more and more recent researches have revealed that early feeding regime and nutrition of calves may produce long-term effects on subsequent growth, health, and milk production performance by epigenetic modifying of gene expression or influencing the establishment of ruminal microbiota (Figure. 1).

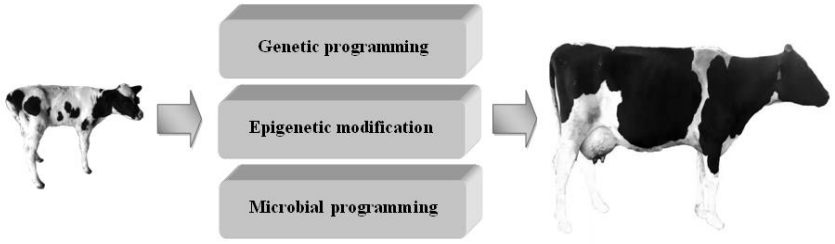

Figure 1. Calves rearing represents a vital determinant of the success of cattle breeding

Epigenetic modification plays a central role in regulating tissue specific gene expression and hence alterations in these processes can induce long-term changes in gene expression and metabolism which persist throughout the life course (Karen et al., 2012). A growing number of studies observe that various dietary factors in early age induce different epigenetic mechanisms that are able to affect growth and health status in later life (Canani et al., 2011). In dairy cattle, increasing nutrient intake from either milk or milk replacer before weaning can increase milk yield during the first lactation, and Soberon et al. (2012) suggested it might be attributed to epigenetic effects of early nutrition.

Early nutrition may also represent a vital determinant of adult growth and health by making an impact on the establishment of rumen microbiota. The postnatal period may be the most feasible "window" for the occurrence of microbial programming effects (Yáñez-Ruiz et al., 2015; Abecia et al., 2013, 2014) (Figure 2). With the development of rumen and the colonization of internal microorganisms, a calf experiences a physiological transition from a pseudo-monogastric animal to a functional ruminant. During this period, any influences from external environment including nutrition could lead to permanent changes in subsequent growth, health, and production performance. Yáñez-Ruiz et al. (2010) reported that the establishment of bacterial community in the rumen of lambs was affected by the diet fed around weaning, and the microbial programming effect exists more than 4 months. De Barbieri et al. (2015) indicated that ruminal bacterial communities of lambs can be altered by the diet of the maternal ewes and lambs or by inoculation treatment, and this alteration persist till 5 months of age.

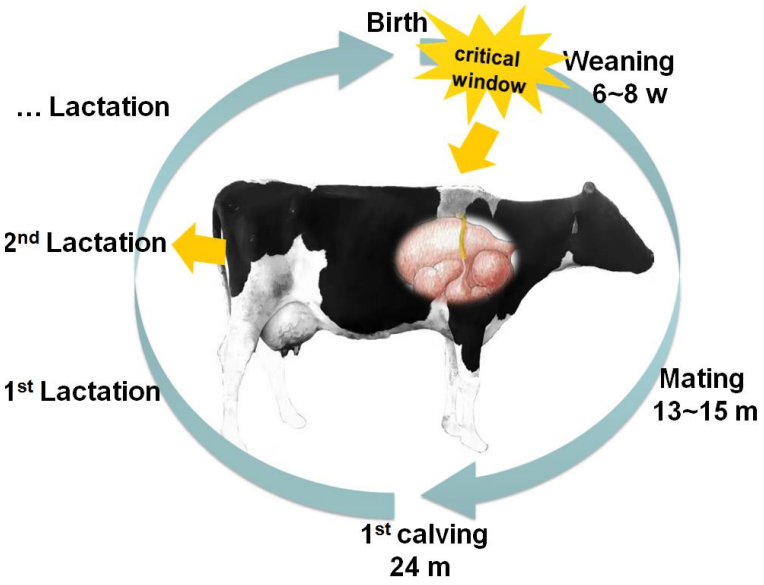

Figure 2. The critical windows for microbial programming

Until now, studies on microbial programming are still limited. We know little about the relationship between liquid feeding regime and nutrition of calves and microbial programming effects. Based on current observation, difference in milk and other liquid feed may induce microbial programming and this phenomenon might be associated with several factors (Figure 3). Firstly, the nutrient level of liquid feeds have effect on solid feed intake. The solid feed is fermented to volatile fatty acids in rumen, which are important in stimulating the growth of rumen epithelium (Flatt et al., 1958; Tamate et al., 1962). In addition, difference in the type or nutrient level of liquid feed may affect the growth and metabolic status of calves, and thereby indirectly influence the development of forestomaches. It was reported that increasing nutrition plane of milk replacer (MR) to calves altered mRNA expression of genes involved in promoting rumen epithelium growth (Naeem et al., 2012). Secondly, a considerable amount of liquid feed may leak into the rumen due to the failure closure of oesophageal groove even in calves those are not identified as ruminal drinkers. Ruminal drinkers refer to calves that have rumen fermentation dysfunction due to ingested milk flowing into rumen instead of abomasum. The amount of liquid feed that enters rumen was estimated to be 17% to 35% of the total intake in bucket-fed calves (Suárez et al., 2007; Labussière et al., 2014; Berends et al., 2015), and 0% to 20% in nipple-fed calves (Ellingsen et al., 2016; Berends et al., 2012). Overfeeding of liquid feeds may exceed the capacity of the abomasum, causing backflow into rumen (Borne et al., 2004; Flor et al., 2012). Thus, differences in liquid feed are possible to directly induce the differences in rumen fermentation environment and bacteria

composition. Thirdly, the concentration of hormones and bioactive factors, such as growth hormone (GH), insulin, insulin like growth factor-1 (IGF-1), and epidermal growth factor (EFG), in liquid feed may play an important role in accelerating gastrointestinal tract development. The high level of biologically active substances in colostrum promote the development, digestion, and absorption ability of the gastrointestinal tract in calves. Insulin, IGF-1 and EGF can contribute to rumen epithelial cell proliferation (Baldwin, 1999; Zitnan et al., 2005). Last but not the least, rumen development is closely associated with that of the small intestine. Górka et al. (2011) observed positive relationships between reticulorumen weight and small intestine weight or brush border enzymes activities, respectively. Thus, different type of liquid feed may affect small intestine development, and indirectly influence the development of forestomaches (Górka et al., 2011).

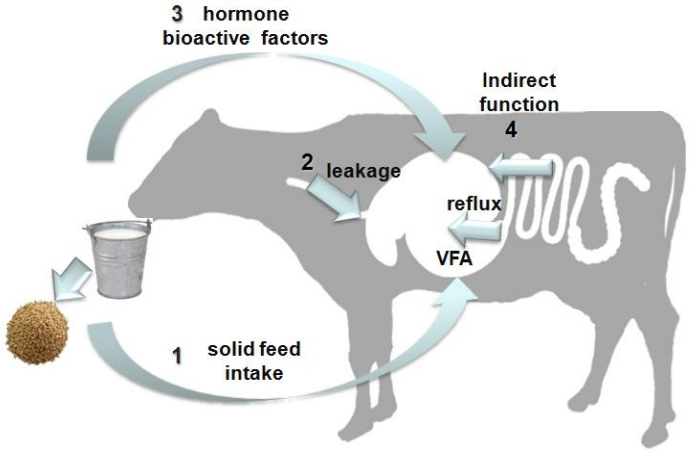

Figure 3. The pathway of liquid feed acting on rumen microbiota

Microbiota-host interaction may act as a primary intervention target for optimizing growth and health in young animals. 16S rDNA amplicon sequencing is a next-generation sequencing methodology that plays an important role in the accurate identification of bacterial isolates, the discovery of novel bacterial genera and species, the detection of uncultivable bacteria (Woo et al., 2008). The development of microbial 16S rDNA gene sequencing and metagenome analysis enables us to learn more about the establishment of rumen microbes and their interactions in host gastrointestinal tract development.

2. The objective and outline of thesis

The objective of this thesis was to investigate the effects of early feeding regime and nutrition on the rumen development of host and establishment of rumen microbes in calves.

Rumen development in newborn calves is one of the most important and interesting areas of calf nutrition. In the chapter 2, we review strategies to promote rumen development in calves. The review contributes a general overview of recent researches concerning two aspects, which are the gastrointestinal (GI) tract development of calves and strategies to promote the physiological and microbiological development of rumen. This article has been published in ***Animals***, 2019, 9 :490.

Whole milk, waste milk and milk replacer are the most commonly used liquid feeds on dairy farms. In chapter 3, we evaluated the effects of these three liquid feeds on growth, rumen fermentation and bacterial community in calves. Fifty-four Holstein calves were randomly allocated to one of three treatments and fed pasteurized waste milk (WM), whole milk (M), or milk replacer (MR). Male calves were slaughtered at age 2 months, and their stomach masses were measured. The female calves were followed for 6 months to determine body weight, blood indices, rumen fermentation, and ruminal bacterial community. The results demonstrated that early life feeding impacted rumen development not only by dry matter intake, but also by the type of liquid feed. Calves fed WM had different rumen fermentation and bacterial community at the weaning period, whereas feeding MR produced a long-lasting effect on the rumen environment. This article has been published in ***Animals***, 2019, 9 :443.

Plant extracts are among the most promising alternatives to antibiotics. Sanguinarine and resveratrol can inhibit a wide range of bacteria, but their effects on rumen development remains to be determined. In chapter 4, we investigate the effects of these two plant extacts on growth, rumen fermentation and bacterial community in calves. Fifty-four female Holstein calves were randomly assigned to three treatments consisting of basal diet alone (MR group) or supplemented with sanguinarine (SAG group) or resveratrol (RES group) at 7 days of age. Body weight was measured at the beginning of the experiment and 2 or 6 months of age. Rumen fluid was sampled at 1, 2, 3, 4, 5, 6, and 9 months of age to monitor rumen fermentation parameters. The results demonstrated that dietary sanguinarine or resveratrol affected rumen fermentation parameters and bacterial community in calves during 3-5 months of age, however, long-term effects of plant extract on rumen environment was not detected at 6 and 9 months of age. This article has been published in ***Animal Feed Science and Technology***, 2019, 251:64-75.

In chapter 5, a general discussion and conclusion were obtained that the type of liquid feed may induce microbial programming effects on calves. Calves fed MR had a different rumen fermentation pattern and bacterial structure at 6 months of age. Although supplementation of

plant extracts did not produce long lasting effects on rumen environment of calves, resveratrol might be used as a promising approach to reduce methane emissions and deserves a further research.

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2

A review: Strategies to promote rumen development in calves

This chapter is adapted from: Review of strategies to promote rumen development in calves. ***Animals***, 2019, 9 (8), 490.

Abstract

Digestive tract development in calves presents a uniquely organized systems. Specifically, as the rumen develops and becomes colonized by microorganisms, a calf physiologically transitions from a pseudo-monogastric animal to a functional ruminant. Importantly, the development of rumen in calves can directly affect the intake of feed, nutrient digestibility and overall growth. Even minor changes in early feeding regime and nutrition can drastically influence rumen development, resulting in long-term effects on growth, health and milk yields in adult cattle. Rumen development in newborn calves is one of the most important and interesting areas of calf nutrition. This paper presents a comprehensive review of recent studies on the gastrointestinal (GI) tract development in calves. Moreover, we also describe the effect of the environment on shaping the GI tract, including diet, feed additives and feeding management, as well as discuss the strategies to promote the physiological and microbiological development of rumen.

Key words: calves; rumen; epithelium; microbiota; diet; feed additives; feeding management

1. Introduction

Rearing healthy calves is very important as it can have a significant impact on their growth and milk production performance in the adult life. Adequate calf development is therefore crucially important for the entire dairy industry. Calves are challenged by a series of stress factors after they are born, including changes in their surroundings. Specifically, the living environment changes from the sterile uterus to the natural outside conditions, in addition to changes in nutrition provided by the mother to the digestion and absorption of feed by calves themselves. However, due to the poor immunity and the incomplete development of the digestive system in young calves, any interference from the external environment or changes to the nutrition can drastically affect the development of calves. Some of the problems include diarrhea, slow weight gain, as well as respiratory tract disease, which can lead to high levels of morbidity and mortality, and pose significant challenges to the breeding.

2. Calf digestive physiology

The digestive organs of animals include the digestive tract and digestive glands. For ruminants, the digestive tract is predominantly composed of the oral cavity, stomaches and intestines, and the digestive glands include liver and pancreas. Digestion begins in the mouth. Ruminants can secrete a large amount of saliva from the salivary glands into the rumen, which plays an important role in ruminal buffering and

fermentation. The saliva of young ruminants contains lipase, however, the lipolytic potency of saliva decreased rapidly with age. In calves fed by conventional methods, the lipase disappeared from saliva during the 3rd month of life (Grosskopf et al., 1965). Compared with monogastric animals, the forestomaches of ruminants has a specialized structure and function, which results in differences in digestion and physiology between ruminants and monogastric animals. Moreover, calves have additional unique esophageal grooves that are present in their digestive tract during their development. Understanding rumen development in newborn calves is one of the most important focus areas of calf nutrition.

2.1 Stomaches

The stomach compartment is comprised of rumen, reticulum, omasum, and abomasum (Figure 4). These four stomach chambers have specific roles in the digestion and absorption of nutrients to ensure efficient feed utilization. The reticulum and the rumen harbor billions of microbes which play an important role in fermentation and digestion of plant materials. The omasum allows for water absorption, and the abomasum is the true stomach, where the acid digestion of feed occurs (Moran, 2002). At birth, the rumen is not completely developed, and significant changes in rumen development have to occur first before the calves can digest dry feed to guarantee their own growth needs. The specific changes include the development of rumen organ, rumen epithelium and establishment of rumen microbiota.

2.1.1 Rumen organ development

The digestive system of young ruminants begins to develop during the embryonic period. For example, the stomach chambers are visible by day 56 in bovine embryos (Warner, 1958). At birth, the weights of reticulorumen, omasum, and abomasum account for 38%, 13%, and 49% of the entire stomach weight, respectively (Davis et al., 1998). By 8 weeks of age, these proportions change to 61.23%, 13.40%, and 25.37% of the stomach weight, respectively (Diao et al., 2017). Finally, at 12-16 weeks of age, they reach 67%, 18%, and 15% of the stomach weight, respectively (Davis et al., 1998; Diao et al., 2017). The abomasum of newborn calves is the only fully developed and functional stomach, and is also the most important digestive organ for calves at birth. The digestion of fat, carbohydrates, and protein is predominantly dependent on the digestive enzymes secreted by the abomasum and small intestine, which is similar to the digestive system in monogastric animals. Over time, with the increase in dry feed intake, the rumen begins to develop and starts to play more important digestive roles.

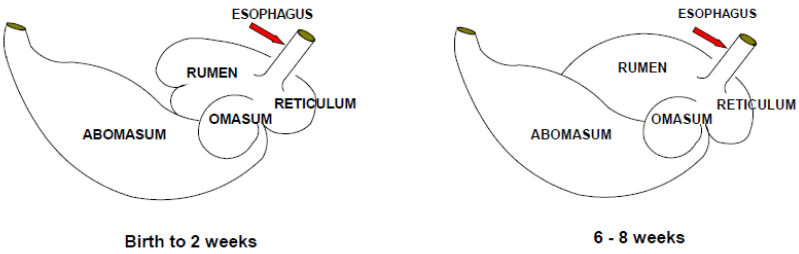

Figure 4. The development of forestomach (Rob Costello, 2005)

2.1.2 Rumen epithelium

The ruminal epithelium performs many important functions and plays the key role in rumen development, including absorption, transportation, short-chain fatty acid metabolism, and protection. The proliferation and growth of rumen squamous epithelium promotes the growth of papillae length and width, and increase the thickness of interior rumen wall (Baldwin et al., 2004). Work by Lesmeister and coworkers (2004) considered the papillae length of the rumen as the most important factor for the evaluation of rumen development, followed by the papillae width and rumen wall thickness. However, papillae per square centimeter are not used as an indicator of rumen development.

Newborn calves have a smooth epithelium with no prominent papillae. Calves fed solely with liquid feed have been shown to have limited rumen development characterized by decrease in rumen weight, papillary growth, degree of keratinization, pigmentation and musculature development (Tamate et al., 1962; Gilliland et al., 1962). Of note, increased intake of solid feed contributes to the rapid development of ruminal fermentation. As calves consume more starter feed, rumen digesta pH decreases, whereas volatile fatty acids (VFA) concentration gradually increases during the first 2 months. The molar proportion of acetate decreases during the first 2 months, and then starts to increase until 9 months of age as forage intake increases (Suarez-Mena et al., 2016; Diao et al., 2017). The presence and absorption of VFAs in the rumen provides chemical stimuli required for proliferation of rumen epithelium (Flatt et al., 1958; Tamate et al., 1962). The absorption of VFA by rumen epithelium is not only by lipophilic diffusion, but also by acid radical ion of VFA/HCO3- anion exchange, nitrate-sensitive VFAs absorption, proton-coupled VFA-transport and electron mediating mechanisms (Aschenbach et al., 2011). Importantly, intraruminal administration of acetate, propionate, and butyrate can stimulate the growth of rumen epithelium in young ruminants, with the effect of butyrate being the most prominent, followed by propionate (Tamate et al., 1962; Baldwin et al., 2004). Studies suggest that rumen papilla proliferation is associated with the

increased blood flow through the rumen wall (Sander et al., 1959; Glauber et al., 1991) and a direct effect of butyrate and propionate on gene expression (Galfi et al., 1991).

Despite many studies indicating that VFA can promote the development of rumen epithelium in vivo, the in vitro results suggest the opposite. For example, butyrate treatment decreases DNA synthesis of rumen epithelial cells in culture (Neogrady et al., 1989), while the proliferation of rumen epithelial cells is inhibited by rumen fluid in vitro (Wang and Jiang, 2010). The divergent in vivo and in vitro response may be linked with an indirect hormonal response to VFA metabolites. Several hormones, such as insulin, pentagastrin, and glucagon, have been implicated as possible VFA mediators that stimulate rumen epithelial proliferation (Sakata et al., 1980; Galfi et al., 1991). Previous study by Baldwin (1999) reported that proliferation rates of rumen epithelial cells induced by insulin, epidermal growth factor, and insulin-like growth factor (IGF-1) were 75%, 97% and 96%, respectively. Importantly, other studies also suggested that insulin, epidermal growth factor, and IGF-1 can overcome the inhibitory effect of butyrate (Galfi and Neogrady, 1989; Baldwin, 1999).

2.1.3 Ruminal microbiota

At birth, the GI tract of young ruminants has generally been regarded as sterile. During the first hours of life, the forestomach becomes rapidly colonized with an abundant bacterial population. However, recent researches reveal that microbial colonization already begin during the fetal development (Stinson et al., 2017; Jaber et al., 2018).

The neonates acquire bacteria from the dam, partners, feed, housing and environment. The early gut microbes of suckled lambs were mainly derived from the mother's teats (43%) and ambient air (28%), whereas those of bottle-fed lambs were dominated by bacteria from the mother's vagina (46%), ambient air (31%), and the sheep pen floor (12%) (Bi et al., 2019). The calf faecal microbiota was highly variable during the first 48 h after birth. The faecal microbiota of calves were more similar to the cow vagina, suggesting that gut microbiota of calves may be derived from the birth canal (Daniela et al., 2019). However, Jaber (2018) indicated that the rectal microbiota of newborn calves was composed of Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, which was resembled dam oral rather than fecal or vaginal microbiota, but included typical intestinal taxa.

Facultative anaerobes such as Streptococcus and Enterococcus are the early colonizers of rumen, which convert rumen to a fully anaerobic environment to promote the rapid establishment of strictly anaerobic bacteria (Jami et al., 2013). By 2 days of age, the rumen microflora reaches 109 cells/mL with strictly anaerobic bacteria being predominantly found in the rumen of lambs (Fonty et al., 1987). The

aerobic and facultatively anaerobic bacteria were 10 to 100-fold lower than the strictly anaerobic bacterial count observed during the first week, which continued to decrease afterwards (Fonty et al., 1987).

Compared to older animals, the abundance of phylum Bacteroidetes was significantly lower in 1-day-old calves and was mainly composed of the genus Bacteroides, whereas older animals were mainly conlonized with Prevotella (Jami et al., 2013). Study by Jami and colleagues (2013) reported that cellulolytic bacteria and other bacterial species important to rumen function can be detected as early as 1 day after birth. The presence of cellulolytic and methanogenic bacteria was observed in lambs at 3 to 4 days of age, and the population of these bacteria reached a level similar to that observed in mature sheep within 7 days of age (Fonty et al., 1987). Thus, the establishment of these rumen bacteria occurs long before young ruminants are given access to concentrated feed or forage.

Dill-McFarland et al. (2017) indicated that calves sampled a few days after weaning had a more diverse rumen community compared to calves sampled during weaning. Several fungal operational taxonomic units (OTUs) observed in weaned calves are also present in adults. As fungi mainly colonize fibrous solids, this may suggest an introduction of forage allows previously low-abundant or transient fungi to persist and multiply.

The rumen bacterial population of 2-week-old calves fed milk replacer (MR) was reported to contain 45 bacterial genera belonging to 15 phyla (Li et al., 2012). Similarly, 47 bacterial genera belonging to 13 phyla were observed in the 3-week-old calves (Malmuthuge et al., 2014). Interestingly, the rumen microbiota of the 2-week-old calves has more heterogeneous microbiota and harbours more abundant yet transient bacterial species and genera compared to calves at 42 days of age (Li et al., 2012). Another study suggested that the diversity and intra-group similarity of rumen microbiota increases with age, suggesting a transition from a heterogeneous and less distinct community to a more homogeneous and diverse mature bacterial population (Jami et al., 2013). This is further supported by a recent study, where gut communities showed higher alpha-diversity but lower beta-diversity with age (Dill-McFarland et al., 2017). Co-habitation facilitates individuals to acquire a shared microbiota (Song et al., 2013). The rumen microbiota was similar in weaned and adult goats that were co-housed pre-weaning (Wang et al., 2016). This may also contribute to a convergence toward a similar microbiota in the adult animals.

The composition of the rumen bacterial community varied significantly among individual calves, suggesting a strong host-microbiota specificity in the rumen (Jami et al., 2013; Li et al., 2012). Similarly, communities of archaea and fungi in rumen varied considerably among individuals (Dill-McFarland et al., 2017; Zhou et al., 2014). This may suggest that the composition of the rumen microbial community is associated with the physiological condition of

the host (Jami et al., 2013). Moreover, work by Mayer et al. (2012) found that fecal microbial composition was more similar between twin calves than between siblings, implying that host genetics partly define individual gut microbial composition.

Additionally, the bacterial composition was different among the gastrointestinal tract regions and between mucosa- and digesta-associated communities (Malmuthuge et al., 2014). Colonization of calf rumen starts early in life with a distinct segregation of bacteria between digesta and epithelial surfaces. Similarly, the methanogen community also varies along the gastrointestinal tract (Zhou et al., 2014). This indicates that previous studies on fecal samples cannot adequately represent the complexity of the gut microbiome. And both mucosa- and digesta-associated communities in rumen play important role in physiological nutrition of calves.

2.2 Esophageal groove

The esophageal groove is one of the unique features inside the GI tract of calves. The majority of the liquid feed, such as colostrum, whole milk and MR, can bypass the rumen, reticulum and omasum, and flow directly into the abomasum as a result of the reflex closure of the esophageal groove (Figure 5). In constrast, water enters the rumen instead of the abomasum, unless it is given immediately after milk. When the esophageal groove is completely closed, milk and other liquid feed can be digested more efficiently in abomasum by avoiding fermentation by the rumen bacteria. When there is an incomplete closure, milk and liquid feed can leak into rumen, disturb rumen microflora, and likely increase the risk of indigestion and diarrhea. Very early studies reported that the reflex closure of the esophageal groove is associated with the stimuli from sucking a teat or the posture of calves when they are sucking (Wise and Anderson, 1939; Wise et al., 1942). Thus, the nipple-feeding method is considered more efficient compared to the bucket-feeding method. However, later studies discovered that the closure of the esophageal groove was independent of the feeding procedure, type of liquid feed and feeding methods (Hegland et al, 1957; Abe et al., 1979). More recent researches estimated the amount of liquid feed that leaks into the rumen is approximately 0-20% of the total intake in nipple-feeding calves (Ellingsen et al., 2016; Berends et al., 2012), and 17-35% in bucket-feeding calves (Suárez et al., 2007; Labussière et al., 2014; Berends et al., 2015).

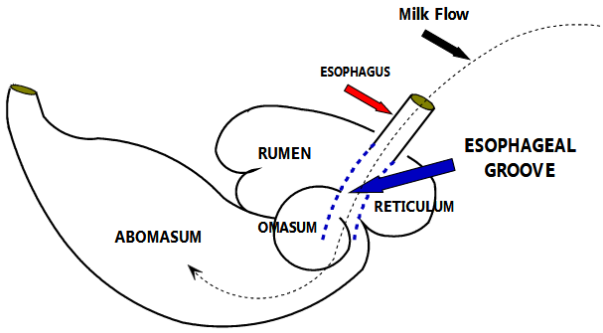

Figure 5. The esophageal groove (Rob Costello, 2005)

2.3 Small intestine

The secretions of digestive enzymes in the small intestine of newborn calves play important roles in feed digestion and absorption of nutrients. For example, work by Drackley (2008) indicated that the digestive enzymes present in postnatal calves enable highly efficient digestion of milk proteins, lactose, and triglycerides.

The liquid feed tends to form clots in the abomasum due to coagulation effects between casein and chymosin/pepsin, however, whey was excluded rapidly from the clot and quickly flow into the small intestine (Heinrichs and Jones, 2003). During the first days of postnatal life, the levels of chymosin and pepsin are sufficient to coagulate casein in lambs and calves (Le et al., 1992). The level of chymosin increases in the first 2 days after birth and then decreases, whereas, levels of pepsin do not change with age. Casein is partially digested by the abomasal pepsin protease and the released polypeptides are further digested by the pancreatic enzymes like trypsin, chymotrypsin, carboxypeptidase, and elastase (Drackley et al., 2008). Pancreatic elastase cleaves globular proteins, like β-lactoglobulin in milk and conglycinin in soybeans (Gestin et al., 1997), and is therefore crucial when calves are fed MR containing plant proteins.

Lactose is hydrolyzed to glucose and galactose by lactase, which are then absorbed by the intestinal epithelium. Importantly, lactase, cellobiase, maltase and trehalase are enzymatically active from the beginning of GI tract development in the bovine fetus (Toofanian et al., 1974). Lactase activity is high at birth and then decreases with age (Le et al., 1992). In contrast, the activity of maltase and pancreatic amylase is low in newborn calves, but the activities of both enzymes markedly increase by 3 weeks of age (Heinrichs and Jones, 2003; Guilloteau and Zabielski, 2005). Importantly, there are no studies reporting sucrose activity in fetal, young or adult ruminants. Therefore, the use of any other sugars other than lactose is limited in calves.

Milk fat is trapped in the clotting curd and digested by pregastric lipase into diacylglycerols and free fatty acids in abomasum. In the

presence of colipase and bile salts, pancreatic lipase hydrolyzes diacylglycerols and the remaining triglycerides to 2-monoacylglycerols and free fatty acids (Drackley et al., 2008). The activity of pancreatic lipase increases with age, but it does not become fully effective in older preruminants since colipase is a limiting factor (Guilloteau et al., 1996). The fatty acids and 2-monoacylglycerols are absorbed by the intestinal epithelial cells, re-esterified to triglycerides, and then packaged to chylomicrons. The chylomicrons are then secreted from the cells into the extracellular space, and transferred to the vena cava by lymphatic system (Drackley et al., 2008).

3. Strategies to promote rumen development

Strategies to promote morphological structure and metabolic function of rumen in preruminants are an ongoing issue which greatly attracts a lot of attention from the scientific community. Numerous studies and approaches attempt to modulate rumen fermentation and microbial community in young ruminants to accelerate rumen development. These approaches include alteration of diet composition and physical forms, addition of new types of feed additives, and differences in the feeding management.

3.1 Diet

3.1.1 Liquid feed

Liquid feed may affect plasma concentration of hormones and growth factors, such as insulin and IGF-1, which play important roles in stimulating proliferation of rumen epithelial cells (Baldwin, 1999; Zitnan et al., 2005). Colostrum contains many biologically active substances, mainly polypeptide growth factors and steroid hormones, including insulin, IGF-1, and transforming growth factor (TGF). Intake of colostrum has been associated with the development, digestion, and absorption ability of the GI tract in the newborn calves (Baldwin, 1999; Zitnan et al., 2005). Moreover, a whole milk calf diet was shown to have a positive effect on milk yield during the first lactation of the adults compared to calves fed MR diet. These results highlight the importance of biologically active milk-borne factors (Moallem et al., 2010).

The composition of MR has significant effects on the establishment of gut microbiota in pre-weaned calves (Badman et al., 2019). The partial replacement of milk protein by plant protein in MR has become common in recent years due to economic reasons (Schoonderwoerd et al. 1989; Campos et al. 1982). However, different sources and replacement proportions of plant protein in milk replacers had different effects on growth performance of preruminant calves (Lalles et al. 1995; Davis et al. 1998). Plant proteins do not clot in the abomasums of calves which may speed up the emptying rate (Petit et al. 1987). Antinutritional factors in plant protein result in gastrointestinal allergy and functional

indigestion (Smith et al. 1975; Dawson et al. 1988; Montagne et al. 2003). Moreover, the unbalanced composition of amino acids in plant protein may induce lack of essential amino acid (Montagne et al. 2001). The sequence order of limiting amino acids for calves fed MR containing soy protein was lysine, methionine, and threonine (Gerrits et al. 1997; Hill et al. 2008). Supplementation of milk replacers containing soy protein with lysine, methionine and threonine (Kanjanapruthipong et al. 1998) or lysine and methionine (Wang et al. 2012) can improve growth performance of calves. The abomasal pH declines more slowly and pH is higher in calves fed MR containing soy flour compared to calves given whole milk (Colvin et al., 1969; Smith and Sissons, 1975; Constable et al., 2005). Decreasing the pH of MR emulsion by addition of an acidifier reduces the pH of digesta in the rumen, reticulum, and omasum. Specifically, pH reduction of MR emulsion was found beneficial for the development of ruminal epithelium (Zhang et al., 2017a). Work by Górka and coworkers (2011a) reported a shorter papillae length of cranial dorsal sac in calves fed MR compared to calves fed whole milk, and noted positive relationships between reticulorumen weight and small intestine weight or with brush border enzymes activities. There is a close relationship between the development of the rumen and the small intestine. Importantly, different types of liquid feed affect the development of small intestine, the intake of solid feed later in life, as well as the growth and metabolic status of calves, thereby indirectly affecting the development of forestomaches (Górka et al., 2011a). Enhancing nutrition level of MR in calves induces changes in the expression of genes coding for proteins directly influencing rumen epithelial growth (Naeem et al., 2012).

Liquid feed may flow into rumen in one of two ways. The first is through insufficient closure of the oesophageal groove. This can occur even in calves that are not clinically defined as rumen drinkers. The amount of leakage liquid to rumen was approximately 17-24% (Labussière et al., 2014), suggesting that liquid feed can directly affect rumen development. The second is through abomasal reflux due to overfeeding liquids beyond the capacity of the abomasum (Borne et al., 2004). Flor et al. (2012) calculated the abomasal volume to be less than 2 L, however, Ellingsen et al. (2016) indicated that voluntary intake of up to 6.8 L of warm whole milk per meal in 3 weeks old calves did not cause abomasal reflux. The occurrence of abomasal reflux may be influenced by several factors, such as feeding techniques or milk type. Abomasal reflux of liquid feed may also alter rumen fermentation.

3.1.2 Water

Water, as an essential nutrient, is extremely important to calves. Unlike liquid feed, water is only capable of entering the rumen (Govil et al., 2017), and thus directly affects rumen development and microbial establishment. Calves receiving drinking water immediately after birth

had higher starter feed intake and body weight (Kertz et al., 1984; Wickramasinghe., et al., 2019). Provision of drinking water to calves positively modulate intestinal microbiota of preweaning calves by increasing the species richness and the abundance of Faecalibacterium and Bifidobacteriumin (Wickramasinghe., et al., 2020).

3.1.3 Starter feed

Feeding readily fermentable carbohydrates to calves increases VFA production in the rumen which is necessary to stimulate the development of rumen epithelium (Heinrichs and Lesmeister, 2004; Mentschel et al., 2001). Calves fed milk-only diet during the first 3 weeks present with a different microbial community in their GI tract and feces compared to calves given milk and solid feed (Guzman et al., 2015). Diets differing in carbohydrate composition lead to differences in rumen fermentation patterns and VFA profiles which may have a variable effect on rumen development (Bannink et al., 2006; Suárez et al., 2006). For example, high concentrations of ruminal ammonia, acetate, propionate, and butyrate were detected in calves fed corn- and wheat-based diets compared to calves fed barley- and oat-based diets. Moreover, the forestomach weight and papillae growth were greater in calves fed corn- and wheat- diets (Khan et al., 2008). The mucosal thickness was greater in calves fed starch- and pectin-based diets compared to calves on NDF-based diets, however, a higher incidence of poorly developed mucosa was observed in calves fed starch-based diet than in animals fed pectin- and NDF-based diets (Suárez et al., 2007). It was reported that the stimulatory effects of VFAs are different, with butyrate being most stimulatory followed by propionate and then acetate (Tamate et al., 1962; Baldwin et al., 2004). Butyrate provides energy required for rumen wall thickening, formation of papillae and stimulating capillary development (Weigand et al., 1975). Butyrate can also increase the blood flow during nutrient absorption and metabolism and can directly affect gene expression in the ruminal epithelium (Baldwin et al., 2004).

Additionally, rumen development can also be affected by the dietary nutrient level. Interestingly, lambs fed high protein diet had a higher concentration of ammonia nitrogen (NH3-N) but lower proportion of total VFA and propionate (Yang et al., 2016). Moreover, study by Shen and coworkers (2004) identified that a high energy diet lead to rumen papillae proliferation which was associated with IGF-1 receptors and increased plasma IGF-1 levels in baby goats. However, excessive consumption of rapidly fermentable starter feed may predispose calves to rumen acidosis. Specifically, it can reduce ruminal pH, decrease rumen motility, and result in keratinization of papillae, causing a decreased in VFA absorption (Beharka et al., 1998; Greenwood et al., 1997; Mentschel et al., 2001).

3.1.4 Forage

Forage is less energy-intensive than starter feed. The low digestibility of forage in the rumen increases gut fill and decrease voluntary intake of concentrated feed by calves, which results in insufficient levels of VFAs required to stimulate rumen growth (Drackley, 2008; Coverdale et al., 2004). However, forage consumption is associated with positive effects of fiber on rumination and salivation in the GI tract (McBurney et al., 1983; Pazoki et al., 2017). The inclusion of forage in the diet increases rumen pH in both pre-weaning and post-weaning calves (Khan et al., 2011; Castells et al., 2013). Importantly, intake of forage was negatively correlated with the severity of subacute ruminal acidosis (SARA), suggesting that a small quantity of consumed forage (0.080 kg/day) can alleviate rumen acidosis in calves (Laarman and Oba, 2011). Forage-fed calves had greater molar proportions of acetate and propionate but lower butyrate and valerate compared to calves that were not fed forage (Terre et al., 2013). Higher acetate and lower propionate concentrations were also found in calves supplemented with straw (Suarez et al., 2006). During weaning transition, feeding dietary forage in calves mitigates ruminal acidosis and induces changes in ruminal bacterial diversity and abundance (Kim et al., 2016). Different forage sources have different effects on stimulating chewing activity and saliva production (Mertens, 1997). Supplementation with NDF from alfalfa hay in starter diet was shown to be more effective than beet pulp in increasing rumen pH and stimulating chewing activity (Maktabi et al., 2016). Additionally, inclusion of forage in the starter feed was positively linked with muscular development of the rumen (Beiranvand et al., 2014; Mirzaei et al., 2015) and morphological appearances of rumen epithelial cells, and caused decreased plaque formation (rumen mucosa containing focal or multifocal patches with coalescing and adhering papillae covered by a sticky mass of feed, hair and cell debris) (Suárez et al., 2007; Beiranvand et al., 2014). Replacing 50% barley or corn with corn silage in the diet given to 10- or 90-day-old calves improved the thickness of the rumen wall, but had no significant effect on the papillae (Sosin-Bzducha et al., 2010). Moreover, the empty rumen weight was greater in calves supplemented with hay compared to calves fed hay-free diet (Castells et al., 2013; Pazoki et al., 2017). A recent meta-analysis indicated that forage consumption can affect starter feed intake and performance in calves, which was modulated by forage level, sources, and physical forms of the starter (Imani et al., 2017).

3.1.5 Physical form

The physical form and particle size distribution of diet exert significant influence on the anatomical and microbial development of rumen (Beharka et al., 1998; Pazoki et al., 2017). For example, calves fed ground diet had shorter papillae with smaller surface area compared

to calves fed the unground diet. Moreover, a decrease in cellulolytic bacteria and an increase in amylolytic bacteria were detected in calves fed the ground diet (Beharka et al.,1998). Consumption of finely ground diets can reduce ruminal pH (Laarman and Oba, 2011) and lead to rumen parakeratosis (McGavin et al., 1976; Greenwood et al., 1997). Given these considerations, 75% of the particles in the starter feed should exceed 1190 μm in diameter (Porter et al., 2007). Work by Lesmeister and Heinrichs (2004) reported that calves fed texturized starter feed containing whole corn had higher ruminal pH compared to calves fed diet with dry-rolling corn, roasted-rolling corn, or steam-flaked corn. Increasing particle score of alfalfa hay from 1 mm to 3 mm can affect non-nutritive oral behaviours in calves fed finely ground starter feed (Nemati et al., 2015). However, studied by Suarez-Mena and coworkers (2015, 2016) suggested that increasing particle size of starter diet by adding whole oat or straw of different lengths had no effect on rumen fermentation and calf development. The effect of the physical form and shape of the diet on calves is closely related to the inclusion rate, source, nutrient matrix and processing method of each ingredient. Importantly, the optimal calf diet specification designed specifically to promote rumen development has not been yet defined.

3.2 Feed additives

3.2.1 Probiotics

Probiotics are viable and beneficial microorganisms that help maintain GI microbial balance and promote rumen development. Feeding probiotics to calves around weaning age may facilitate the development of rumen bacterial communities and help calves with a transition from liquid feed to dry feed and forage (Krehbiel et al., 2003; Hong et al., 2005). Fermentation products of Saccharomyces cerevisiae have been shown to positively influence ruminal microbiota and improve ruminal morphology (Kuma et al., 1994; Xiao et al., 2016). Specifically, effects of Bacillus licheniformis, Saccharomyces cerevisiae and their compounds can increase nitrogen utilization of the rumen microbial community and affect the fermentation pattern which was shown to be beneficial for growth of fattening lamb (Jia et al., 2018). Oral dose of M. elsdenii NCIMB 41125 given to calves at 14 days of age increased ruminal butyrate, reticulorumen weight and papillae growth, suggesting an improvement in epithelial metabolism (Muya et al., 2015). Oral administration of Bacillus subtilis natto was shown to aid the development of rumen bacterial communities by increasing the growth of cellulolytic bacteria in calves after weaning (Yu et al. 2009).

However, feeding probiotics to calves has not always been shown to exert positive effects on the development of celluloytic bacteria. For example, oral administration of a mixture of Lactobacillus plantarum and Bacillus subtilis in calves affected the denatured gradient gel

electrophoresis (DGGE) fingerprint of the 16S ribosomal RNA genes, and reduced the number of Ruminococcus albus (Zhang et al., 2017b). In contrast, other studies reported that pH and enzymatic activities of rumen fluid were unaffected by three kinds of probiotic feeding in newborn calves (Agarwal et al., 2002). Supplementation with Candida tropicalis had no effect on the morphology of the forestomach and enzymatic activities of ruminal digesta (Wang et al., 2018). Rumen and papillae measurements of Holstein bull calves were not affected by dietary inclusion of Aspergillus oryzae fermentation extract (Yohe et al., 2015). Overall, the effects of probiotics on rumen development in calves are inconclusive, and frequently driven by differences in viable probiotic bacterial numbers, probiotics species, administration methods, and health status of animals.

3.2.2 Effects of VFAs

VFAs are the primary products of rumen fermentation and contribute to rumen epithelium development in calves. Butyrate and propionate are considered to be the main stimulators of rumen epithelium development. Butyrate is the preferable energy source for rumen epithelial cells (Lane and Jesse, 1997). It can stimulate rumen epithelium proliferation and promote papillae growth, which is far more effective than propionate (Tamate et al., 1962; Mentschel et al., 2001). Previous studies suggested that infusion of sodium propionate or sodium butyrate greatly promotes the development of the rumen papillae in calves (Tamate et al., 1962; Mentschel et al., 2001; Guilloteau et al., 2004). Supplementation of MR with sodium butyrate was associated with increased reticulorumen weight and increased length and width of papillae (Górka et al., 2009; 2011a; 2011b). Another study showed that calves receiving a blend of short and medium chain fatty acids as monoglycerides (0.2%) in MR had less degenerative tissue accumulation and a higher number of cytoplasmic protrusions on the exposed horn surfaces (Ragionieri et al., 2016).

Branched-chain VFAs (BCVFA), such as isobutyrate, isovalerate and 2-methylbutyrate, are naturally derived from the catabolism of branched-chain amino acids. Adequate levels of BCVFA are essential for the growth of some cellulolytic bacteria and digestion of structural carbohydrates in the rumen (Cummins and Papas, 1985; Liu et al., 2008ab). Dietary supplementation with isobutyrate and isovalerate can accelerate the growth of calves by improving ruminal fermentation, rumen enzyme activities and growth of cellulolytic bacteria (Liu et al., 2016; Wang et al., 2014).

3.2.3 Plant extracts

There are many studies focused on evaluating the potential of plant extracts as alternatives to feed antibiotics and growth promoters in ruminant nutrition. Plant extracts have been shown to favorably affect

rumen microbiota (Giannenas et al., 2011), and modulate ruminal fermentation (Hristov et al., 1999; Calsamiglia et al., 2007; Macheboeuf et al., 2008) in ruminants. However, studies evaluating how plant extracts affect rumen development in young ruminants are limited. Recent study revealed that Aloe barbadensis was beneficial in increasing total VFA concentration and bacterial count in cross-bred calves (Kuma et al., 2018). Supplementation with mulberry leaf flavonoids increased α-amylase activity in ruminal digesta and protease activity in abomasal digesta in calves (Wang et al., 2018). Caraway and garlic can improve rumen fermentation parameters by increasing total VFAs, increasing rumen pH and decreasing rumen ammonia in growing buffalo calves (Hassan and Abdel-Raheem, 2013). Thyme and cinnamon essential oils were shown to decrease the molar proportion of acetate and lower the ratio of acetate to propionate, as well as increase the level of propionate in Holstein calves consuming a high-concentrate diet. Finaly, cinnamon essential oil was shown to increase rumen molar concentration of butyrate (Vakili et al., 2013). Plant extracts are among the most promising alternatives to antibiotics due to their extensive biological effects, and can be used in calf feed to prevent diarrhea. However, the efficacy of plant extracts is subjected to a series of factors, including the composition of active components, addition levels, and physiological status of animals. The using of different types of plant extracts at various inclusion rate in diet deserves further researches. Moreover, effects of plant extracts on the colonization of microbial populations remains to be determined in calves.

3.3 Feeding management

Weaning age can influence the development of rumen in preruminants. For example, calves weaned at 6 weeks of age had longer and wider papillae compared to calves weaned at 9 weeks of age (Žitňan et al., 1999). In early-weaned calves, the ruminal pH, molar proportion of acetate and the ratio of acetate to propionate were lower, but the molar proportion of propionate and butyrate were greater (Mao et al., 2017). The β-diversity of ruminal microbiota shifts rapidly in calves weaned at 6 weeks, while a more gradual shift is observed in calves weaned at 8 weeks (Meale et al., 2017). The colonization pattern substantially differs between newborn goats reared naturally with the dam and those reared artificially with MR. Additionally, a higher bacterial diversity was observed in natural-fed goats (Abecia et al., 2017). The total rumen bacterial population of lambs grazing at pasture with the nursing mother was lower compared to lambs weaned at 21 or 35 days of age, whereas, methanogens and protozoa population were lower in early-weaned lambs compared to grazing lambs (Ji et al., 2016). Additionally, pair-housed calves were shown to consume more solid feed at earlier age compared to calves housed individually (de Paula Vieira et al., 2010; Costa et al., 2015).

However, studies evaluating the effect of feed management are

inconsistent. Kehoe and coworkers (2007) reported that weaning age and milk feeding frequency had no effect on rumen papillae length, width or rumen wall thickness. Different weaning methods (conventional weaning or concentrate-dependent weaning) result in similar rumen development (Roth et al., 2009). The development of ruminal microbiome was not affected by the weaning strategy, and there was no effect of gradual or abrupt weaning (Meale et al., 2016). Moreover, the time of forage provision (at 3 or 15 days of age) also had no effect on rumen fermentation parameters in calves (Wu et al., 2017).

4. Conclusions

To sum up, it is beneficial for rumen development for calves fed high-quality liquid feed rich in biologically active substances. Minimize the use of soy protein or appropriate acidification of MR may alleviate gastrointestinal epithelium lesions. Feeding readily fermentable carbohydrates to calves to increase VFA production can stimulate rumen development. A pellet or texturized starter feed is superior to a finely ground meal. Meanwhile, providing high-graded forage to calves, such as alfalfa hay, can reduce the occurrence of rumen acidosis and papillae keratinization. Moreover, additives can be used in calf feed due to their potential advantages in rumen manipulation, however, the types and the optimal inclusion rate deserved further researches. More importantly, there is no fixed pattern of calf feed. The diet compositions and nutrient specifications should be matched with the feeding program and management to better promote rumen development.

Rumen is the most unique part of the GI tract in ruminants. As the rumen develops and becomes colonized by microorganisms, a calf physiologically transitions from a pseudo-monogastrics to a functional ruminant. The development of rumen in calves can directly affect feed intake, nutrient digestibility and eventual growth of calves. Any changes in early feeding regime and nutrition can influence rumen development, and thus, lead to long-lasting effects on subsequent growth, health, and milk production performance. Studies indicated that diets can modify the establishment of bacterial community in lambs during weaning, which can persist for 4 to 5 months (Yáñez-Ruiz et al., 2010; De Barbieri, 2015). The postnatal period is frequently referred to as the most sensitive window for rumen manipulation (Yáñez-Ruiz et al., 2015; Abecia et al., 2013, 2014), although studies evaluating microbial programming are still limited. The majority of published studies focus on rumen organ development, rumen fermentation parameters, morphology, and changes in the population of cellulolytic bacteria. With the development of microbial 16s rDNA gene sequencing and metagenome analysis, additional studies will likely reveal the interactions between host GI tract development and establishment of rumen bacteria.

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3

Article 1: Early feeding regime of waste milk, milk and milk replacer to calves has different effects on rumen fermentation and bacterial community

Promoting rumen development has always been a key target of calf nutrition. During the postnatal period, the establishment of rumen microbiota is vulnerable to changes in the external environment. Among them, liquid feed is the main feed for calves and may induce long-term effects on the calves. In this chapter, we evaluated the effects of mostly commonly used liquid feeds on rumen development in calves.

This chapter is adapted from: Early feeding regime of waste milk, milk and milk replacer to calves has different effects on rumen fermentation and bacterial community. ***Animals*.** 2019, 9, 443.

Abstract

We investigated the effects of different types of early feeding on rumen fermentation parameters and bacterial community in calves. Fifty-four Holstein calves were randomly allocated to one of three treatments and fed pasteurized waste milk (WM), whole milk (M), or milk replacer (MR) during 7 to 63 days of age. Male calves (n = 3 per group) were slaughtered at age 2 months, and their stomach masses were measured. The female calves (n = 15 per group) were followed for 6 months to determine body weight, blood indices, rumen fermentation, and ruminal bacterial community. At age 2 months, the average daily gain of the MR group was less than that of the other two groups, whereas starter intake, stomach mass, and rumen mass as a percentage of stomach mass were lower for the WM group. The concentration of total volatile fatty acids was greater for the MR group, whereas the isovalerate molar proportion was greater for the WM group. The concentrations of growth hormone, human epidermal growth factor, insulin-like growth factor 1, and the growth hormone to insulin ratio were greater, but the levels of insulin and nonesterified fatty acids were lower, for the WM group. The bacterial community in the rumen of the WM group differed from that of the other groups. At age 6 months, the ruminal propionate molar proportion was lower, but the ruminal pH and acetate/propionate ratio were greater, for the MR group. No difference was observed for the average daily gain, and blood indices, but the serum urea nitrogen concentration was lower in the MR group. Early life feeding impacted rumen development not only by dry matter intake, but also by the type of liquid feed. Calves fed WM had different rumen fermentation and bacterial community at the weaning period, whereas feeding MR produced a long-lasting effect on the rumen environment.

Keywords: early feeding regime, growth, blood indices, rumen fermentation, bacterial community

1. Introduction

Whole milk (M), waste milk (WM), and milk replacer (MR) are the most common liquid feeds for calves on dairy farms. Waste milk is comprised of colostrum, transition milk, milk obtained from mastitic cows, and milk from cows treated with antibiotics. Growth performance has been shown to be similar for calves fed WM and M (Chardavoyne et al., 1979; Kesler, 1981), whereas the growth rate of calves fed MR is influenced by the ingredient composition and nutrient intake of the MR (Dawson et al., 1988; Li et al., 2008). As most liquid feed flows directly into the abomasum upon suckling action which closes the oesophageal groove, the intestine, rather than the rumen, is the major digestion site for pre-weaned calves. Thus, previous research mainly focused on the effects of different liquid feed on intestinal microbiota and development. Colostrum was shown to have a positive effect on gastrointestinal tract

development and function in calves, not only through the provision of nutrients, but also due to the high concentration of growth factors and biologically active peptides (Guiloteau et al., 1997; Blum and Hammon, 2000). Calves fed pasteurized WM had a more diverse bacterial community in feces compared to those of calves fed non-pasteurized waste milk (Edrington et al., 2012). Calves fed MR containing soy flour had a less acidic abomasal environment (Smith and Sissons, 1975; Constable et al., 2005) and slower intestinal development(Górka et al., 2011).

As the rumen develops and is colonized by microorganisms, a calf physiologically transits from a non-ruminant to ruminant state. The postnatal period may be the most critical window for rumen manipulation, and early feeding regime may lead to permanent changes in rumen microbial composition (Yáñez-Ruiz et al., 2015; Abecia et al., 2013). Diets could modify the establishment of bacterial community of lambs around the weaning, and this modification persists over 4 months (Yáñez-Ruiz et al., 2010). De Barbieri et al. (2015) also found that ruminal bacterial communities of lambs can be altered by the diet of the maternal ewes and lambs or by inoculation treatment , and the difference exists till 5 months of age. Thus, we evaluate the effects of the most common liquid feeds on calf ruminal development, fermentation, and bacterial community at weaning period, and then we followed them up to 6 months of age to determine the persistence of any effects.

2. Materials and methods

2.1 Animals, Treatments, and Management

Fifty-four Holstein calves (n = 45, female; n = 9, male) were recruited from the Yin Xiang Dairy Farm (Shandong, China) and the average age of calves was 2.8 days. Calves were fed 4 L of frozen-thawed standard colostrums by esophageal feeder immediately after they were born. And then calves were accustomed to bucket feeding with M ,WM or a transition MR, respectively. The feeding amount is 5 L per day. The transition MR are composed of 2.5 L of M and 2.5 L of MR. At 7 days of age, the experiment began and calves were randomly assigned to one of three treatments and fed M, pasteurized WM, or MR, respectively. The WM was pasteurized at 65°C for 30 min. The MR was reconstituted as an emulsion (12.5%, w/v) in warm (40°C) water. A mobile calf feeding equipment (MilchMobil, Foerster-Technik, Germany) was used to control the feeding amount and temperature (37-39°C) of these three types of liquid feed to calves. Calves were bucket fed twice a day. The amount of M, WM, or MR fed to the calves was listed in Table S1. None of the liquid feed was left. A pelleted feed and water were provided *ad libitum* to the calves from 14 days of age on. No forage was supplemented during 7 to 63 days of age. Calves were weaned gradually from 60 to

63 days of age. From 7 to 63 days of age, calves were raised in separate calf hutches (1.2 × 1.8 m). From 64 days to 6 months of age, calves were raised together according to their treatment in naturally ventilated barns (15 calves per barn). A pelleted feed and forage were provided separately *ad libitum* from age 2 to 6 months. Milk and pasteurized WM were sampled biweekly. MR (patent number, CN02128844.5) was purchased from the Beijing Precision Animal Nutrition Center, Beijing, China, The nutrient profiles and antibiotic residuals of M, WM and MR are listed in Table S2. The ingredient composition and nutrient profile of the pelleted feed and forage are presented in Table S3. The experiment began when calves were 7 days of age and terminated when they were 6 months old. The protocol (protocol number: AEC-CAAS-2015-01) was approved by the Animal Ethics Committee of the Chinese Academy of Agriculture Science (Beijing, China).

Table S1. The feeding amount of liquid feed during 7 to 63 days of age

Age	L
7~14 d	5.0
14~28 d	5.4
28~42 d	6.2
42~60 d	7.3
61 d	5.5
62 d	3.7
63 d	1.8

Table S2. Chemical analysis of the milk, waste milk and milk replacer emulsion.

Chemical analysis	Milk[a]	Waste Milk[a]	Milk replacer emulsion[b]
Crude Protein (%)	3.3±0.27	4.29±0.61	2.71±0.01
Crude Fat (%)	4.25±0.25	3.91±0.56	1.90±0.04
Crude Lactose (%)	4.47±0.27	3.85±0.52	3.54±0.00
Gross energy (Mcal/kg) [c]	6.40	6.40	3.97
Gentamycin (mg/L)[d]	-	0.067±0.042	-
Cephalexin (mg/L)[d]	-	-	-
Cefapirin (mg/L)[d]	-	-	-
Ceftiofur (mg/L)[d]	-	-	-

[a] Data were measured by lab analysis.

[b] Data were calculated based on chemical analysis of milk replacer powder. The patent number of milk replacer is CN02128844.5. The crude protein, fat, lactose, NDF, ADF, Ash, Calcium and total phosphate of milk replacer powder on dry matter basis is 22.93%, 16.02%, 30.0%, 5.07%, 1.52%, 4.3%, 0.9% and 0.49%, respectively. The ingredient compositions are extruded soy flour, whole milk powder, whey permeate (lactose 80%), starch dextrin (amylodextrins), calcium carbonate, dicalcium phosphate, lysine, methionine, threonine, vitamin premix, trace minerals premix and additives.

[c] GE (Mcal/kg)=0.057×CP%+0.092×Fat%+0.0395×Lactose% (NRC, 2001)

[d] The type of drugs were provided by veterinary staff.The limits of quantification (LOQ) for Gentamycin, Cephalexin, Cefapirin and Ceftiofur is 4, 2, 1 and 50 μg/L, respectively. "-" means the content was below LOQ.

Table S3. Composition and chemical analysis of the concentrate feeds (Dry matter basis)

	Concentrate feed		Forage[d]	
Ingredients	0-3 Month	4-6 Month	Alfalfa hay	Oat hay
Corn (%)[a]	55.65	56.5		
Soybean Meal (%)[a]	26.2	23.15		
Extruded Soy (%)	7	0		
Wheat bran (%)	3.9	10		
DDGS (%)	3	6		
Calcium Carbonate (%)	2.25	2.30		
Phospate Dicalcium (%)	0.6	0.55		
Salt (%)	0.4	0.5		
Premix (%)[b]	1	1		
Items[c]	0-3 Month	4-6 Month	Alfalfa hay	Oat hay
Dry Matter (%)	87.94	87.77	90.22	89.50
Crude Protein (%)	20.00	18.00	18.77	7.18
Crude Fat (%)	3.86	3.21	2.32	2.27
NDF (%)	9.79	11.41	35.31	50.33
ADF (%)	3.77	3.95	26.22	32.36
Crude Ash (%)	6.94	6.99	7.35	5.57

Calcium (%)	1.00	1.00	1.28	0.32
Total Phosphate (%)	0.45	0.45	0.26	0.20
Salt (%)	0.47	0.58	-	-

[a] The screen size of grinding mill is 2.5 mm for corn and 3.0 mm for soybean meal.

[b] provided per kg of basal diet: 10 000 IU vitamin A, 1 500 IU vitamin D, 60 IU vitamin E, 1.5 mg vitamin B1, 8.2 mg vitamin B2, 2.0 mg vitamin B6, 3.6 mg vitamin K, 1.0 mg folic acid, 0.1 mg biotin, 49.5 mg niacin, 60.0 mg D-pantothenic acid, 10.2 mg Cu, 20 mg Fe, 140 mg Zn, 140 mg Mn, 2.0 mg I, and 0.44 mg Se.

[c] Data were caculated by the measured value of each ingredients.

[d] The composition of forage is 50% alfalfa and 50% oat hay during 2-3 months, and 30% alfalfa and 70% oat hay during 4-6 months.

2.2Sampling and Measurements

2.2.1 Growth Performance

Body weight of calves was measured before the morning feeding when the calves were 7, 14, 28, 49, 58, 90, 120, 150 and 180 d old. The offered pelleted feed and refusals were weighed daily to calculate feed intake on the 7 consecutive days prior to weaning at 60 d old (female calves, n = 15 per each treatment).

2.2.2 Blood Metabolite and Hormones Measurements

Blood was sampled before the morning feeding at 60 and 180 days of age (female calves, n = 10 per treatment). The blood samples were centrifuged at 1350 x g for 20 min at 4°C. The serum was decanted and stored at -20°C before being used to determine the concentration of serum urea nitrogen (SUN), nonesterified fatty acids (NEFA), growth hormone (GH), insulin (INS) and insulin-like growth factor-1 (IGF-1). The SUN and NEFA concentrations were determined using a Model 7600 automatic biochemical analyzer (Hitachi, Tokyo, Japan). The insulin concentration was determined using radioimmunoassay kit (Beijing SINO-UK Institute of Biological Technology, Beijing, China) and a GC-911-γ-Radiation immunity arithmometer (Zhongke zhongjia Scientific Instruments Co., Ltd., Anhui, China). The concentration of GH and IGF-1 were measured using ELISA kits (Beijing SINO-UK Institute of Biological Technology, Beijing, China) and a Stat Fax 2100 microplate reader (Awareness Technology Inc, Palm City, FL, USA).

2.2.3 Assessment of Rumen Organ Development

Male calves were slaughtered at 58 days of age (n = 3 per treatment). Stomach compartments were collected, emptied, cleaned with saline, drip dried, and weighed. Stomach mass of calves was calculated as a percentage of live weight, and the wet mass of each stomach compartment was calculated as a percentage of the total weight

of the four stomachs.

2.2.4 Rumen Fermentation Parameters Measurements

Ruminal liquid was sampled at 60 and 180 days of age from calves fed WM, M or MR, respectively (WM2, M2, MR2, WM6, M6, MR6) (female calves, n = 8 per treatment). Ruminal liquid was collected 2 to 3 h after the morning feeding using an oral stomach tube (female calves, n = 8 per each treatment). A stainless steel pipe was inserted into the rumen and then a 150 ml syringe was used to aspirate rumen fluids. The first 100 mL of fluid was discarded and the remainder saved. Each sample was individually filtered through a double layer of gauze and collected into a clean tube. Samples (15 mL) placed individually into vacuum tubes were kept at -20°C to analysis of volatile fatty acids (VFA) and ammonia nitrogen (NH_3-N). Other tubes containing 2 mL of the ruminal liquid samples were placed into liquid nitrogen prior to analysis of cellulolytic bacteria and bacterial community. Ruminal pH values were determined immediately after sampling using a digital Basic PB-20 pH meter (Sartorius AG, Göttingen, Germany). The NH_3-N concentration was determined using the phenol hypochlorite colorimetric method (Broderick and Kang, 1980). The VFA concentration was determined using an SP-3420 gas chromatograph system (Beijing Analytical Instrument Factory) according to Zhang and colleagues (2016).

Figure S1. The picture of oral stomach tube

2.2.5 Identification of the Rumen Bacterial Community

DNA Extraction, PCR Amplification, and Illumina Sequencing

Total DNA was extracted from 1 mL of each ruminal liquid sample using QIAamp Fast DNA Stool Mini kit (QIAGEN, Germany) according to the manufacturer's instructions. Amplification by PCR was conducted with the 515f/806r primer set that amplifies the V4 region of the 16S rDNA gene (515F: 5'-GTG CCA GCM GCC GCG GTA A-3'; 806R 5'-XXX XXX GGA CTA CHV GGG TWT CTA AT-3')(Caporaso et al., 2011). The reverse primer containing a 6-bp error-correcting barcode unique to each sample PCR amplification were performed in 30 μL mixture containing 15 μL of 2× Phusion High-Fidelity PCR Master Mix (New England Biolabs, USA), 2 μM of forward and reverse primers, 10 ng DNA template, and 2 μL of HPLC-grade water. The cycling conditions consisted of an initial cycle of 98°C for 1 min, followed by 30 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 30 s, and a final cycle of 72°C for 5 min. The PCR products were excised from a 2% (w/v) agarose gel and purified using a GeneJET Gel Extraction kit (Thermo Scientific). Illumina paired-end sequencing libraries were constructed using NEBNext DNA sample preparation kit (New England Biolabs, USA). DNA quality was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) followed by quantification on the Qubit 2.0 Fluorometer (Life Technologies). Sequencing of amplified bacterial 16S rRNA gene fragments was performed using an IlluminaHiSeq2500 platform (Novogene Bioinformatics Technology Co., Ltd., Beijing, China).

Bioinformatic Analysis

Pairs of reads from the original DNA fragments were assigned to each sample according to the unique barcodes and were merged using FLASH (v1.2.7). Raw tags were quality filtered using QIIME (v1.7.0) (Caporaso et al., 2010). Chimeric sequences were identified by comparison with those in the Gold database (version microbiomeutil-r20110519, http://drive5.com/uchime/uchime_download.html) and then removed using UCHIME (Edgar et al., 2011). Sequences were assigned to operational taxonomic units (OTUs) at a 97% identity threshold using UPARSE (v7.0.1001) (Edgar, 2013). Taxonomic classifications were assigned using the SILVA SSURef database release 123 and the mothur-based implementation of the RDP classifier (Wang et al., 2007). Alpha diversity as indicated by the Chao1 and Shannon indices were analyzed with QIIME and displayed using the ggplot2 package in R (Wickham, 2009). Principal coordinate analysis (PCoA) based on unweighted UniFrac distance was calculated using QIIME.

2.3Statistical Analysis

The data for growth, blood indices, stomach mass and rumen fermentation were analyzed using the general linear model (GLM) procedure of SAS (SAS Version 8.01, SAS Institute, Inc., Cary, NC,

USA). Duncan's multiple range tests were conducted when a significant difference was detected among means. Chao1 and Shannon indices were analyzed by *t*-test to determine whether differences existed within community diversity. An analysis of similarity randomization test (ANOSIM; Clarke, 1993) was used to calculate *P*-value and to determine whether differences existed in microbial composition between the groups. Different bacterial taxa at the genus level were determined with *t*-test, and only those with a relative abundance > 0.1% in at least one sample were visualized using perl-SVG module (Scalable Vector Graphics, v5.18.2). A value of $P < 0.05$ reported statistical significance. Non-parametric Spearman rank correlation coefficient analysis was conducted using the PROC CORR procedure of SAS (SAS Version 8.01, SAS Institute, Inc., Cary, NC, USA) to detect possible relationship between rumen fermentation parameters and bacterial community. The threshold of statistically significance at $P < 0.05$ was described to illustrate relationships. The correlation matrix was visualized using the corrplot package in R (Wei, 2013).

3. RESULTS

3.1 Early Feeding Regime Affected Growth, Rumen Fermentation, and Blood Indices in 2-Month-Old Female Calves, and Rumen Organ Development in Male Calves.

The body weight and average daily gain (ADG) of female calves fed MR was significantly less than calves fed WM or M at 58 days of age ($P = 0.0273$; $P = 0.0004$, respectively). Starter intake for the WM group was less than that for the M and MR groups ($P = 0.0249$; Figure 6). The concentration of total volatile fatty acids (TVFA) was greater for the MR group than for the other groups ($P = 0.0215$). The molar proportion of isovalerate was greater for the WM group than for the other groups ($P = 0.0256$; Table 1). The NEFA concentration was less for the WM group than for the other groups ($P = 0.0037$). The concentrations of GH, IGF-1, and the GH/insulin ratio were greater for the WM group than for the other groups ($P = 0.0005$; $P < 0.0001$; $P < 0.0001$; $P < 0.0001$, respectively), and the insulin concentration was less for the WM group than for the other groups ($P = 0.0141$; Table 2). Additionally, the stomach mass as a percentage to live weight of male calves was less for the WM group than for the M and MR groups ($P = 0.0395$). The wet mass of rumen expressed as a percentage of the total weight of the four stomachs was significantly less ($P = 0.0065$), whereas that of abomasum was significantly greater ($P = 0.0056$) for the WM group than for the other groups (Table 3).

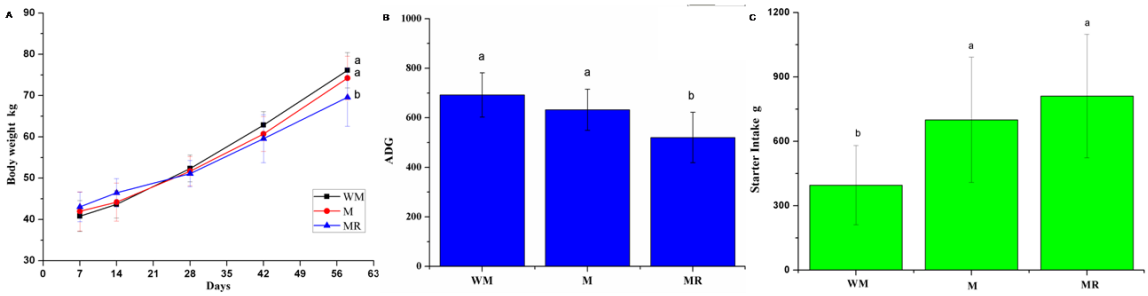

Figure 6. Growth performance of pre-weaning calves (female, n = 15 per each treatment). (A) Body weight of calves during 7 to 58 days of age; (B) ADG of calve during 7 to 58 days of age; (C) Starter intake of calves during 54 to 60 days of age; Data are expressed as means±standard deviation. [ab]Mean values with different superscripts are different at $P < 0.05$ according to Duncan's multiple-range test.

Table 1. Rumen fermentation parameters for 2-month-old female calves (female, n = 8 per each treatment).

Items	Treatment[3]			SEM	*P* value
	M	WM	MR		
Ph	6.15	6.28	5.67	0.174	0.0651
NH_3-N(mg/dL)	20.15	30.08	19.17	4.337	0.1787
TVFA (mmol/L)[1]	40.45[b]	39.21[b]	62.13[a]	5.269	0.0215
Acetate (%)[2]	47.15	49.48	44.39	2.693	0.4483
Propionate (%)[2]	35.29	31.03	38.87	2.39	0.1051
Butyrate (%)[2]	10.59	11.95	11.49	1.863	0.8731
Isobutyrate (%)[2]	0.91	1.57	0.79	0.314	0.2055
Valerate (%)[2]	4.37	2.94	3.02	0.608	0.2041
Isovalerate (%)[2]	1.69[b]	3.03[a]	1.45[b]	0.399	0.0256
Acetate/propionate	1.36	1.77	1.2	0.204	0.1624

[1] TVFA: total violate fatty acids. [2] Acetate(%), Propionate(%), Butyrate(%), Isobutyrate(%), Valerate(%), and Isovalerate(%) imply the molar proportion of each to that of the TVFA. [3] WM: waste milk, M: whole milk, MR: milk replacer. [ab] Mean values within a row with different superscripts differ.

Table 2. Blood metabolites and hormones concentrations for 2-month-old female calves (female, n = 10 per each treatment).

Items	Treatment[7]			SEM	*P*-value
	M	WM	MR		
SUN (mmol/L)[1]	5.65	5.64	5.03	0.5232	0.6866
NEFA (mmol/L)[2]	0.49[a]	0.37[b]	0.44[a]	0.0232	0.0037
GH (ng/mL)[3]	3.93[b]	4.70[a]	3.94[b]	0.1385	0.0005
Insulin (IU/mL)	18.53[a]	9.17[b]	15.29[ab]	2.1231	0.0141
GH/insulin [4]	0.26[b]	0.63[a]	0.27[b]	0.0482	<0.0001
IGF-1 (ng/mL)[5]	168.46[b]	219.31[a]	167.44[b]	7.1208	<0.0001

[1] SUN: serum urea nitrogen; [2] NEFA: nonesterified fatty acid.; [3] GH: growth hormone; [4] GH/insulin: growth hormone/insulin ratio; [5] IGF-1: insulin-like growth factor; [6] WM: waste milk, M: whole milk, MR: milk replacer; [ab] Mean values within a row with different superscripts differ.

Table 3. Stomach development for 2-month-old calves (male, n = 3 per each treatment).

Items	Treatments[3]			SEM	*P* value
	M	WM	MR		
Stomach mass[1]	2.12[a]	1.26[b]	2.19[a]	0.21	0.0395
Rumen %[2]	51.89[a]	42.05[b]	55.05[a]	1.88	0.0065
Reticulum %[2]	8.53	7.75	8.83	0.50	0.3603
Omasum %[2]	11.13	13.28	10.55	0.93	0.1746
Abomasum %[2]	28.45[b]	36.93[a]	25.58[b]	1.58	0.0056

[1]Stomach mass is expressed as a percentage of the live weight of calves.
[2]Individual mass of each stomach compartment as percentages of the total weight of the four stomachs,
[3]WM: waste milk, M: whole milk, MR: milk replacer. [ab] Mean values within a row with different superscripts differ.

3.2Early Feeding Regime Affected Rumen Fermentation and Blood Indices in 6-Month-Old female Calves

No difference was observed in the body weight of calves at 90, 120,

150 and 180 days of age. ADG of calves during 58 to 180 days of age was similar among groups (Figure 7). The molar proportion of ruminal propionate in the MR group was less than in the WM and M groups (P = 0.0221), whereas the ruminal pH and acetate/propionate ratio in the MR group were significantly greater than in the other groups at 6 months of age (P = 0.0038; P = 0.0055, respectively; Table 4). No difference was observed in the blood indices expect for the SUN concentration, which was less in the MR group (P = 0.0303; Table 5).

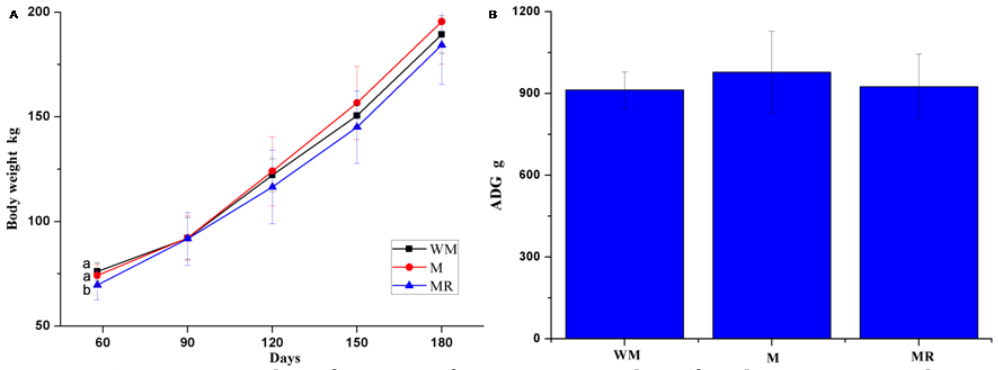

Figure 7. Growth performance of post-weaning calves (female, n = 15 per each treatment). (A) Body weight of calves during 58 to 180 days of age; (B) ADG of calve during 58 to 180 days of age; Data are expressed as means±standard deviation. [ab]Mean values with different superscripts are different at P < 0.05 according to Duncan's multiple-range test.

Table 4. Rumen fermentation parameters for 6-month-old female calves.

Item	Treatment[3]			SEM	*P* value
	M	WM	MR		
Ph	6.36[b]	6.38[b]	6.71[a]	0.077	0.0038
NH_3-N(mg/dL)	10.22	9.67	11.92	1.063	0.3484
TVFA (mmol/L)[1]	70.29	71.82	61.5	4.888	0.3263
Acetate (%)[2]	61.86	60.92	66.72	1.698	0.0683
Propionate (%)[2]	25.49[a]	25.52[a]	19.14[b]	1.63	0.0221
Butyrate (%)[2]	8.76	9.46	10.18	0.759	0.458
Isobutyrate (%)[2]	0.83	0.9	1.08	0.129	0.398
Valerate (%)[2]	1.66	1.57	1.21	0.125	0.0565
Isovalerate (%)[2]	1.41	1.63	1.67	0.148	0.445

Acetate/propionate	2.55[b]	2.44[b]	3.70[a]	0.255	0.0055

[1] TVFA: total violate fatty acids; [2]Acetate(%), Propionate(%), Butyrate(%), Isobutyrate(%), Valerate(%), and Isovalerate(%) imply the molar ratio of each to that of TVFA; [3] WM: waste milk, M: whole milk, MR: milk replacer; [ab] Mean values within a row with different superscripts differ.

Table 5. Blood metabolites and hormones of 6-month-old female calves (female, n = 10 per each treatment).

Items	Treatment[6]			SEM	*P*-value
	M	WM	MR		
SUN (mmol/L)[1]	8.17[a]	8.09[a]	6.78[b]	0.3896	0.0303
NEFA (mmol/L)[2]	0.42	0.41	0.41	0.0162	0.8815
GH (ng/mL)[3]	4.10	4.58	3.98	0.2647	0.2449
Insulin (IU/mL)	14.95	14.53	13.82	1.8822	0.9128
GH/insulin [4]	0.30	0.36	0.33	0.0447	0.6181
IGF-1 (ng/mL)[5]	177.06	186.54	195.43	19.2450	0.7977

[1] SUN: serum urea nitrogen; [2] NEFA: nonesterified fatty acid.; [3] GH: growth hormone; [4] GH/insulin: growth hormone/insulin ratio; [5] IGF-1: insulin-like growth factor; [6] WM: waste milk, M: whole milk, MR: milk replacer; [ab] Mean values within a row with different superscripts differ.

3.3Ruminal Bacterial Community

A total of 2,504,870 sequences were generated from 47 samples, with an average of 51,661 retained sequences following quality filtering and chimera removal for each sample. The average length of the retained sequences was 253 base pairs. The overall number of operational taxonomic units (OTUs) detected was 2,337 based on a 97% nucleotide sequence identity between reads (Table S4). The rarefaction curves indicated that the number of each sequence had approached the saturation plateau, which indicated that sufficient coverage of all OTUs had been obtained so as to accurately describe the bacterial diversity (Figure S2).

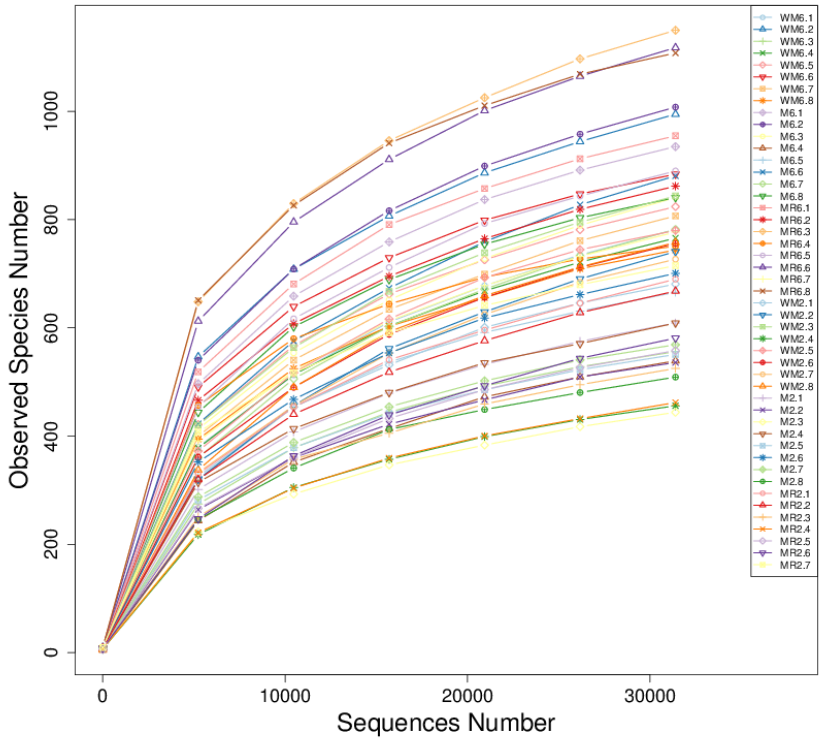

Figure S2. Rarefaction curves

Table S4. Details on number of sequencing reads, retained reads following quality filtering and chimera removal, and operational taxonomic units (OTU)

Sample [a]	Sequencing reads	Retained reads	Retained%	OTU
M2.1	55,053	53892	97.89	734
M2.2	50,635	49595	97.95	674
M2.3	32,538	31732	97.52	444
M2.4	54,692	53534	97.88	738
M2.5	53,722	49371	91.90	666
M2.6	50,825	47701	93.85	830
M2.7	49,441	48278	97.65	679
M2.8	45,441	43803	96.40	610
M6.1	60,588	59201	97.71	1102

M6.2	63,527	61810	97.30	1222
M6.3	62,998	61348	97.38	1018
M6.4	49,421	47630	96.38	688
M6.5	45,601	44089	96.68	805
M6.6	64,261	62928	97.93	1068
M6.7	63,106	59497	94.28	978
M6.8	40,441	39443	97.53	990
MR2.1	64,541	62984	97.59	856
MR2.2	63,708	62320	97.82	820
MR2.3	64,233	62746	97.68	659
MR2.4	53,208	51939	97.62	574
MR2.5	47,127	45847	97.28	674
MR2.6	57,232	54912	95.95	734
MR2.7	53,201	51940	97.63	825
MR6.1	50,357	49284	97.87	1091
MR6.2	53,489	52503	98.16	1015
MR6.3	56,875	55667	97.88	1319
MR6.4	38,622	38048	98.51	836
MR6.5	52,131	50810	97.47	1048
MR6.6	54,267	51907	95.65	1274
MR6.7	56,845	55467	97.58	943
MR6.8	48,499	47321	97.57	1254
WM2.1	45,773	44691	97.64	849
WM2.2	59,889	58676	97.97	916
WM2.3	52,488	50980	97.13	1004
WM2.4	48,265	46836	97.04	542
WM2.5	50,875	46772	91.94	942
WM2.6	53,727	52325	97.39	934
WM2.7	63,651	62065	97.51	924

WM2.8	47,983	46970	97.89	930
WM6.1	54,214	52947	97.66	954
WM6.2	55,600	52522	94.46	1152
WM6.3	42,230	41224	97.62	682
WM6.4	58,265	54001	92.68	956
WM6.5	57,173	55817	97.63	993
WM6.6	48,872	47793	97.79	1024
WM6.7	58,957	57770	97.99	979
WM6.8	50,283	49137	97.72	892

[a]M2.1-2.8, WM2.1-2.8, MR 2.1-2.8: Sample from 2-month-old calves fed whole milk, waste milk and milk repalcer (n = 8 per each treatment); M6.1-6.8, WM6.1-6.8, MR 6.1-6.8: Sample from 6-month-old calves fed whole milk, waste milk and milk repalcer (n = 8 per each treatment)

Bacteroidetes was the most predominant phylum in all samples (65.61%), followed by *Firmicutes* (20.6%), *Proteobacteria* (9.99%), *Tenericutes* (1.20%), *Spirochaetes* (1.09%), *Cyanobacteria* (0.44%), *Actinobacteria* (0.32%), *Synergistetes* (0.15%), *Euryarchaeota* (0.08%), *Fibrobacteres* (0.11%) and unclassified others (0.43%) (Figure 8). Rumen microbiota was more diverse in the WM2 group compared to that in the M2 group, as indicated by Chao 1 (Figure 9A; $P = 0.0391$). No significant difference was observed in Shannon diversity (Figure 9B).

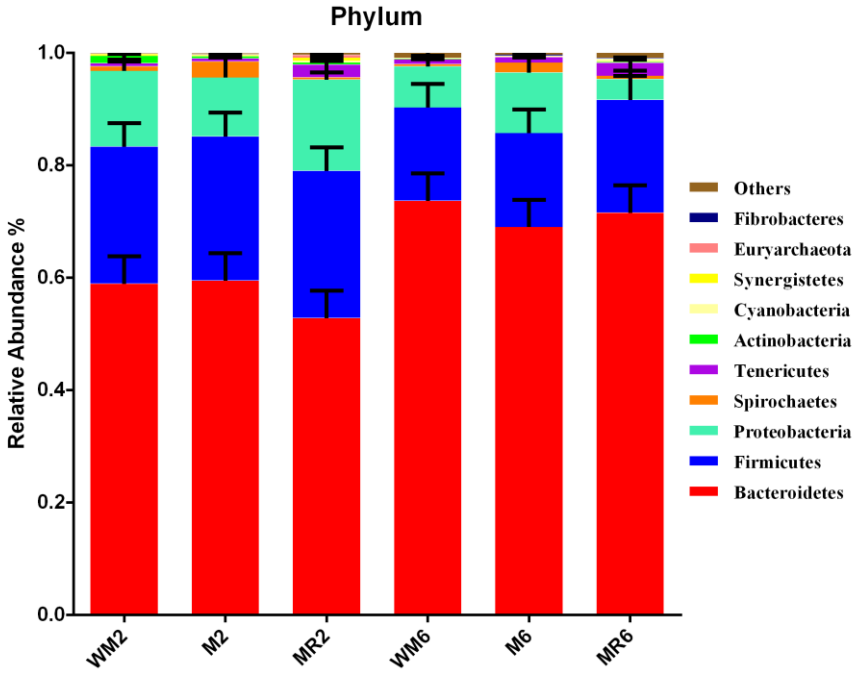

Figure 8. Relative abundance of rumen bacteria at the phylum levels. The 10 most abundant phyla are presented. Each bar represents the average relative abundance of each bacterial taxon within a treatment group. Data are expressed as means±SEM. WM2, M2 and MR2: Rumen liquid was sampled at age 2 months from calves fed waste milk, milk and milk replacer, respectively. WM6, M6 and MR6: Rumen liquid was sampled at age 6 months from calves fed waste milk, milk and milk replacer, respectively.

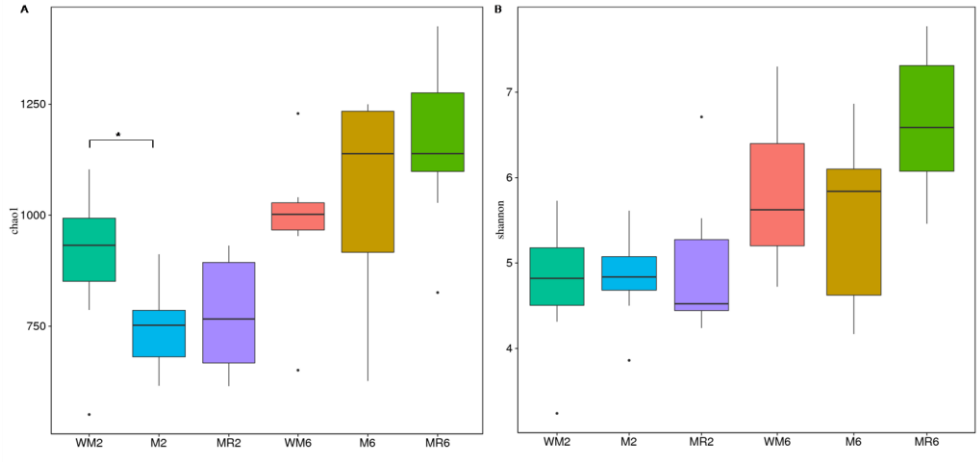

Figure 9. Alpha-diversity measures indicated by the (A) Chao1 richness and (B) Shannon diversity . The horizontal lines within boxes represent the median, and the tops and bottoms of the boxes represent 75th and 25th quartiles, respectively. Outliers are plotted as individual points. *Significant difference was detected between treatment groups at $P < 0.05$ according to *t*-test. WM2, M2 and MR2: Rumen liquid was sampled at age 2 months from calves fed waste milk, milk and milk replacer, respectively. WM6, M6 and MR6: Rumen liquid was sampled at age 6 months from calves fed waste milk, milk and milk replacer, respectively.

The principal coordinate analysis (PCoA) that used unweighted Unifrac distances indicated a separation between 2-month-old calves and 6-month-old calves. The bacterial community structure of WM2 was distinct from M2 (R = 0.2874, $P = 0.001$, ANOSIM) and MR2 (R = 0.1895, $P = 0.018$, ANOSIM), and that of MR 6 was distinct from M6 (R = 0.4955, $P = 0.003$, ANOSIM) and WM6 (R = 0.4542, $P = 0.005$, ANOSIM) (Figure 10).

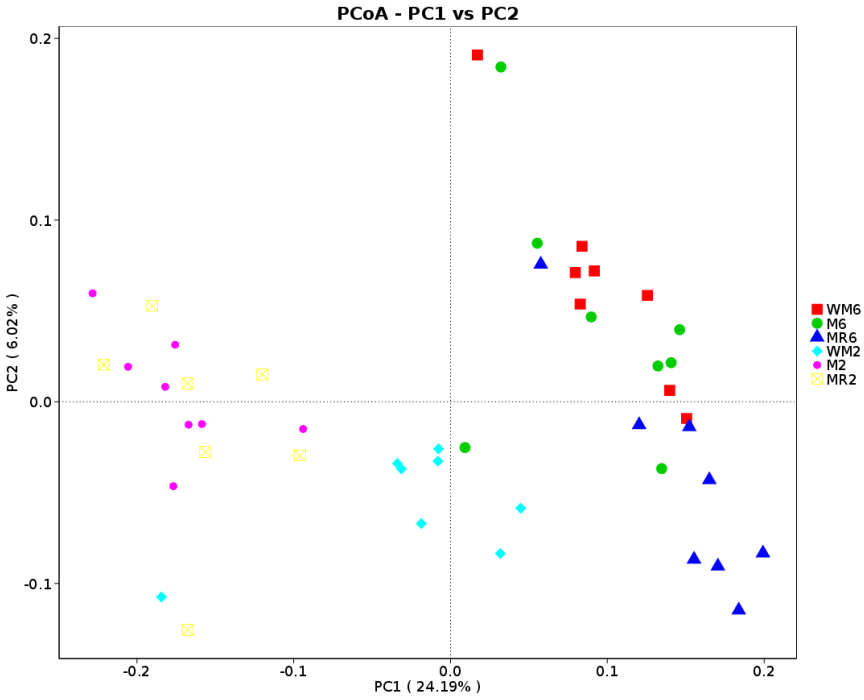

Figure 10. Beta-diversity measures of the rumen bacterial community by principal coordinate analysis based on unweighted UniFrac distances. WM2, M2 and MR2: Rumen liquid was sampled at age 2 months from calves fed waste milk, milk or milk replacer, respectively. WM6, M6 and MR6: Rumen liquid was sampled at age 6 months from calves fed waste milk, milk or milk replacer, respectively.

Significant differences in the rumen bacterial taxa at the genus level were determined using *t*-test for the different paired groups of calves. The genera Rikenellaceae RC9 gut group, *Butyrivibrio 2*, and *Prevotellaceae UCG-003* were more abundant, whereas the genus *Prevotella 7* was less abundant in the WM2 group than those in the M2 or MR2 group, (Figure 11AB). Rikenellaceae RC9 gut group, *Selenomonas 1*, *Prevotellaceae UCG-003*, Lachnospiraceae NK3A20_group and Ruminococcaceae NK4A214 group were more abundant in MR6 group, whereas *Prevotella 7* and *Succinvibrionaceae UCG-001* were overrepresented in WM6 group (Figure 11C). Rikenellaceae RC9 gut group, *Prevotellaceae UCG-003*, *Prevotellaceae UCG-001* and Ruminococcaceae NK4A214 group were more abundance in MR6 group, whereas *Prevotella 7* and *Succinvibrionaceae*

UCG-001 were more abundant in M6 group (Figure 11D). No difference was observed between M2 and MR2, M6 and WM6.

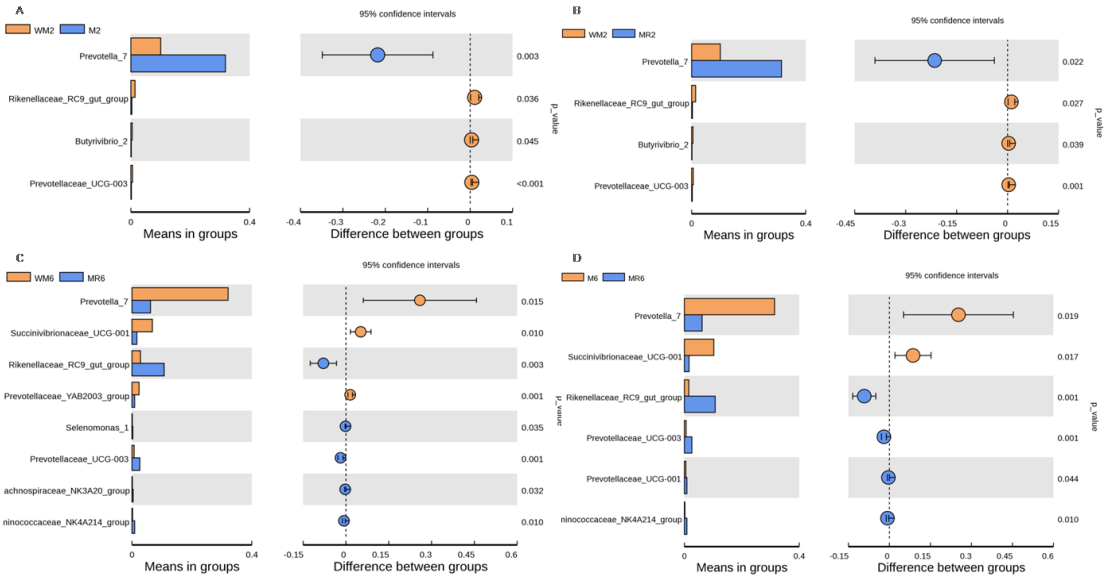

Figure 11. Taxonomic comparisons of the bacterial community at the genus level. Only different bacterial taxa ($P < 0.05$, *t* test) with relative abundance > 0.1% in at least one sample were presented. (A) the WM2 and M2 groups; (B) the WM2 and MR2 groups; (C) the WM6 and MR6 groups; (D) the M6 and MR6 groups. WM2, M2 and MR2: Rumen liquid was sampled at age 2 months from calves fed waste milk, milk or milk replacer, respectively. WM6, M6 and MR6: Rumen liquid was sampled at age 6 months from calves fed waste milk, milk or milk replacer, respectively.

3.4Correlation Analysis

Correlations between rumen fermentation parameters and bacterial species are presented in Figure 12. Even though less bacterial species were correlated to the pH, TVFA, acetate, propionate, butyrate, valerate and A/P ratio in 2-month-old calves than those in 6-month-old calves, more bacterial species were correlated to the NH_3-N, isobutyrate and isovalerate in 2-month-old calves.

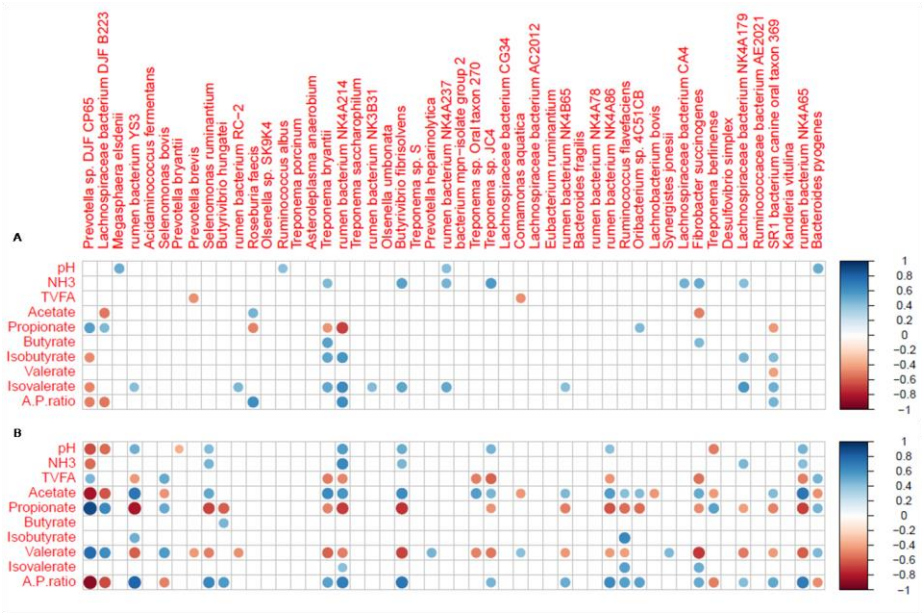

Figure 12. Spearman's rank correlations between rumen fermentation parameters and relative taxa abundance in (A) 2-month-old and (B) 6-month-old calves. Only bacterial species with relative abundance > 0.01% in at least one sample were included in the correlation analysis. Correlations with a threshold of statistical significance at P <0.05 were visualized. The degree of correlation was indicated by the size of circle. The blue color represents a positive correlation and the red color represents a negative correlation.

4. Discussion

In calves that have not been weaned, most of their liquid feed intake bypasses the rumen and enters the abomasum directly because the oesophageal groove is closed. Abe et al. (1979) reported that the reflex closure of the oesophageal groove occurs efficiently independently of the feeding method (nipple-feeding or bucket-feeding method) when calves are familiar with either method. However, Wise et al. (1984) indicated that reticular groove reflex is more efficient when calves suck nipples than those drink from buckets. Changes in feeding method from bucket to floating nipple were successful to improve plasma metabolic and endocrine profiles of ruminal drinking calves (Herrli-Gygi et al., 2006). More recent research found that a considerable amount of liquid feed may leak into the rumen, and the amount has been estimated to be 17% to 35% of the total intake in bucket-fed calves (Suárez et al.,2007; Labussière et al., 2014; Berends et al., 2015), whereas that in nipple-fed calves ranges from 0% to 20% of total milk intake (Ellingsen et al., 2016; Berends et al., 2012). Bucket feeding method has been widely used because it is labor saving and easy to operate. However, it brings more risk to the occurrence of

rumen drinking, which may lead to ruminal fermentation disorders and metabolic acidosis (Gentile et al., 2004). Subacute ruminal acidosis (SARA) is characterized by a sustained depress in ruminal pH below pH 5.6 (Plaizier et al., 2008) or 5.8 (Zebeli et al., 2008) in dairy cows. However, rumen pH of dairy calves averaged between 5.09~5.31 (Suárez et al., 2007) and 5.19~6.16 (Khan et al., 2008), which is lower than those in mature cows. It is mainly because preweaning calves are generally encouraged to maximum dry feed intake to promote rumen development. Additionally, the leakage of liquid feed to rumen may result in bacterial fermentation of milk and lead to ruminal acidosis. In this trial, the ruminal pH was within the normal pH scope of calves, which may suggest no abnormal rumen function occurs to experimental calves. Additionally, milk proteins may rot in the rumen and essential amino acids can be destroyed by rumen microbiota, which may induce indigestion and diarrhea, subsequently reducing growth performance of calves.

Waste milk is comprised of colostrums, transition milk, milk obtained from mastitic cows and milk from cows treated with antibiotics. We assessed the nutrient profile of the three liquid feeds fed to our calves and found the average crude protein of the WM (4.29%) was greater than that of the M (3.3%). Thus, the content of milk protein that entered the rumen might be greater in the WM group than in the M group. Branched-chain VFA (BCVFA) are synthesized by microorganisms in the rumen via oxidative deamination and decarboxylation of branched-chain amino acids (Rosener and Uhlenhopp, 1987). Isobutyrate, isovalerate, and 2-methylbutrate are generated from valine, leucine and isoleucine, respectively. The relative greater concentration of isovalerate in the rumen of the WM group may be associated with the greater protein content of WM. Supplementation of isovalerate increased the population of *Butyrivibrio fibrisolvens* in calves and steers feeds (Liu et al., 2014; Liu et al., 2016). This was confirmed by our result that *Butyrivibrio fibrisolvens* was positively correlated with the concentration of isovalerate in 2-month-old calves. Additionally, the population of *Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogenes* were also linearly increased with increasing isobutyrate or isovalerate supplementation (Wang et al., 2015; Liu et al., 2014; Liu et al., 2016). No differences in abundance of these bacteria were detected in this trial. It is mainly because these bacteria firmly adhere to plant tissues when initiating cellulose degradation (Miron et al., 2001), however, we sampled ruminal liquids, which did not survey the bacterial populations that attached to feed particles. Malmuthuge (2014) reported that the bacterial composition was different between digesta-associated communities and those attached to the epithelium. Our results of ruminal liquids may not adequately represent the complexity of the rumen microbiome. Both digesta and epithelium-associated communities in rumen deserve further investigation. Deng et al. (2017) reported the number of OTUs in the

rumen digesta of calves fed pasteurized waste milk was higher than that in calves fed untreated whole milk. We observed that, compared to untreated whole milk, feeding pasteurized waste milk increased ruminal bacterial chao1 richness. This may be associated with the concentration of isovalerate in the rumen, as a greater population of total bacteria was detected in the rumen of steers fed isovalerate (Liu et al., 2014). However, no difference was observed in the shannon diversity among groups.

Extraruminal effects of BCVFA have been detected by feeding BCFA to dairy cows: the level of GH increased and that of insulin and of NEFA decreased (Fieo et al., 1984; Towns and Cook, 1984; Liu et al., 2009). A similar result was observed in our trial that the greater concentration of ruminal isovalerate might induce increases in serum GH and IGF-1, but decreases in insulin and NEFA in the WM2 group. BCVFA receptors are believed to be present in ruminal and hepatic membranes and might perturb the function of hormone-regulated system such as those involving insulin and IGF-1 (Andries et al., 1987; Hamamdzic, 1989). Additionally, bovine colostrum is characterized by a high level of IGF-1, which was found to be resistant to pasteurization (Elfstrand et al., 2002; Jansson et al., 1985). Thus the bioavailability of these IGF-1growth factors in colostrums may contribute to greater concentrations of serum IGF-1 in calves fed WM. Rauprich et al. (2000) reported that calves fed colostrums had greater plasma IGF-1 than those fed MR. Insulin, IGF-1 and EGF have been implicated as possible mediators of rumen epithelial cell proliferation and thus play an important role in accelerating rumen development in calves (Baldwin, 1999; Zitnan et al., 2005).

Waste milk contains milk obtained from the sick cattle treated with antibiotics. The concentration of antibodies in WM may be higher than those in W and MR. However, pasteurization of WM may inactivate the activities of antibodies, which limit their beneficial effects on the health of calves (Godden et al., 2003). Nevertheless, the concentration of antibiotics residues in waste milk cannot be reduced by pasteurization (Jorgensen et al., 2006). It is thus likely that a substantial number of bacteria may be sensitive to the presence of antibiotic residuals in WM, and that may lead to a distinctive rumen bacterial community of calves in the WM2 group. Antibiotic susceptibilities of ruminal bacteria have been determined previously (Nagaraja and Taylor, 1987). Chlortetracycline, oxytetracycline, tylosin and monensin had a strong inhibitory effect on in vitro cellulose digestibility and VFA production in mixed rumen cultures (Baldwin et al., 1982). Monensin and virginiamycin can alter in vivo rumen microbial populations (Weimer et al., 2008; Coe et al., 1999). Feeding calves milk with very low concentrations of ampicillin, ceftiour, penicillin and oxytetracycline affected the composition of microbial population in feces (Van Vleck et al., 2016). The concentration of antibiotics residues in waste milk cannot

be reduced by pasteurization (Jorgensen et al., 2006). It is thus likely that a substantial number of bacteria may be sensitive to the presence of antibiotic residuals in WM, and that may lead to a distinctive rumen bacterial community of calves in the WM2 group. In this experiment, gentamycin was the only antibiotic residues detected in waste milk and the concentration was 0.067±0.042 mg/L (mean±SD). The decreased relative abundance of *Prevotella 7* in WM2 group might be explained by its susceptibility to gentamycin, which was supported by a recent research that gentamycin inhibited 90% of *Prevotella intermedia* (Anumala et al., 2019). A decreased abundance in genus *Prevotella* was also reported in calves fed waste milk containing 0.024 mg/L of penicillin, 0.025 mg/L of streptomycin, 0.10 mg/L of tetracycline, and 0.33 mg/L of ceftiofur (Li et al., 2019).

Feeding calves a limited amount of liquid nutrition to encourage rumen development is an effective strategy that promotes the transition from pseudo-monogastric animals to functional ruminants. The nutrient value and compositions of MR are different from WM and M. The ratio of protein to energy in the diet affect nutrition utilization and growth performance of calves. Increasing the protein level of MR from 16 to 26% linearly increased growth rates of calves (Blome et al. 2003). However, an increased BW, net gain and ADG were observed in calves fed isocaloric MR containing 22% CP than those fed 18% and 26% CP (Li et al., 2008). This may be explained by the differences in protein sources, because the higher inclusion rate of soy protein in 26% CP treatment might result in a lower N utilization (Li et al., 2008). Hill et al. (2008) indicated preruminant calves had limited ability to digest vegetable proteins. The digestibility of milk protein ranged from 90% to 97% (Holmes and Davey, 1976), whereas that of MR containing soy protein ranged from 70% to 78% (Li et al., 2008). The lower amount of digestible protein found in the MR fed to the calves prior to weaning in our study would account for their slower growth rate. Additionally, supplementing extra fat in milk replacer increased BW gains of calves during cold weather (Jaster et al., 1990; Jaster et al., 1992). Our experiment was conducted in winter and the average temperature of calf house ranged from -5 to 5 °C. The decreased growth rate of calves fed MR can also be attributed to the lower fat content in MR.

Starter feed intake is a good indicator of rumen development. The weaning criterion was defined as the calf age at a minimum daily starter intake of ≥ 1000 g for 3 consecutive days (Sanei et al., 2012). In this trial, we observed that calves in WM consumed less starter feed than the above weaning requirement, which was in accordance with the data of stomach weight obtained from male calves. Starter feed intake was similar for calves in M and MR groups, however, much greater TVFA was observed in MR group. There are two possible reasons. Firstly, leakage volume of MR to rumen may be different from that of W or WM. Previous researches indicated that milk, solutions containing sodium salts or copper sulphate would promote closure of the groove

(Ross, 1931; Watson, 1944), however, other studies reported the closure of the groove was not affected by the nature of liquid feeds (Orskov et al., 1970, Hegland et al., 1957). Furthermore, the type of liquid feed may affect the occurrence of abomasal reflux. The presence of soy protein in MR may impair passage of ingesta in the abomasum, which may contribute to a greater volume of abomasal reflux. Górka (2011) reported that MR containing soy protein had a negative effect on calves' growth, metabolic status and small intestine development, and these effects may indirectly inhibit rumen development. Secondly, the higher TVFA in calves fed MR are probably due to the higher starter feed intake. However, due to lack of valid data on feed intake, it is difficult to disassociate its effect on rumen environment from that of liquid feed. Starter feed intake is generally considered as the main stimulator of rumen development, however, our results suggest that rumen development is also closely associated with liquid feed type and its composition.

The postnatal period may be the most critical window for rumen manipulation, and early feeding regime may lead to permanent changes in rumen microbial composition (Yáñez-Ruiz et al., 2015; Abecia et al., 2013). It was reported that the structure of the bacterial community establishing in lambs was affected by the diet fed around weaning, and this effect persists over 4 months (Yáñez-Ruiz et al., 2010). Ruminal bacterial communities of lambs can be modified by the diet of the maternal ewes and lambs or by inoculation treatment, and this modification exists till 5 months of age (De Barbieri et al., 2015). We noted a long-lasting effect on rumen fermentation and bacterial community at age 6 months for the calves fed MR. The greater pH and the ratio of acetate to propionate in MR6 group might have a close relationship with rumen bacterial composition. The species *Prevotella sp. DJF CP65* is a member of the genera *Prevotella 7*, and its presence positively correlated with the molar proportion of propionate and negatively correlated with the pH value, the molar proportion of acetate and acetate/propionate ratio. As the abundance of *Prevotella 7* was significantly less in the rumen of calves fed MR, it might explain the differences found for the rumen fermentation parameters. Conversely, a significant increase in the presence of the species *rumen bacterium NK4A214* might contribute to the increased pH, acetate/propionate and decreased propionate. Moreover, although all the feeding, management and field conditions for calves was the same during 2 to 6 months, barn effects may exist to influence rumen environment of calves, which impairs the imprinting evidence from early feeding regime in this trial.

5. Conclusion

Early feeding regime impacted rumen development not only by dry matter intake, but also the type of liquid feed. Calves fed WM showed a distinct structure of bacterial community at 2 months of age,

but this difference diminished at 6 months of age. Calves fed MR had a greater concentration of TVFA at 2 months of age, which may induce a long-lasting effect on rumen environment.

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Article 2: Sanguinarine and resveratrol affected rumen fermentation parameters and bacterial community in calves

The microbial community composition and fermentation profile are differently affected by the nature of the liquid feed. This leads to the idea of microbial programming by early dietary interventions. Sanguinarine and resveratrol are potentially effective in manipulating rumen microbial ecosystem owing to their extensive antimicrobial activities.

This charpter is adapted from: Sanguinarine and resveratrol affected rumen fermentation parameters and bacterial community in calves. ***Animal Feed Science and Technology***, 2019, 251, 64-75.

Abstract

Plant extracts can be used in calf feed as alternatives to antibiotics, but their effects on colonization of microbial populations remains to be determined. Thus, we evaluated the effects of dietary plant extracts on rumen fermentation parameters and rumen bacterial community in calves, and we followed them up to 9 months of age to determine the persistence of any effects. Fifty-four female Holstein calves were randomly assigned to three treatments consisting of basal diet alone (MR group) or supplemented with 0.05 mg/kg sanguinarine (SAG group) or 4.0 mg/kg resveratrol (RES group) at 7 days of age. Plant extracts were supplemented until 6 months of age. Body weight was measured at the beginning of the experiment and 2 or 6 months of age. Rumen fluid was sampled at 1, 2, 3, 4, 5, 6, and 9 months of age to monitor rumen fermentation parameters. Rumen samples at 3 and 6 months of age were used to analyze the bacterial community by amplicon sequencing. The copy number of *Desulfovibrio* and methanogenic archaea was determined using droplet digital PCR. The results demonstrated that ADG of calves was similar among groups during 7 d-2 m, 2 m-6 m and 7 d-6 m, respectively. At 3 months, ruminal pH was lower in the SAG and RES groups than in the MR group. The concentration of total volatile fatty acids (TVFA) was greater in the SAG group than in the MR group. At 4 months, the ammonia nitrogen (NH_3-N) concentration and the molar proportion of butyrate were lower in the SAG group than in the other two groups. The molar proportion of acetate and the ratio of acetate to propionate (A:P ratio) were lower in the RES group than in the SAG group. The molar proportion of valerate was greater in the RES group than in the other groups. At 5 months, the NH_3-N concentration was lower in the SAG group than in the other groups. The molar proportion of valerate was lower in the RES and SAG groups than in the MR group. No differences were observed in rumen fermentation parameters among groups at 1, 2, 6, and 9 months of age. The observed species, Chao1, and abundance-based coverage estimator (ACE) values were greater in the MR group than in the other groups at 3 months of age. The community structure of bacteria in the MR group was distinct from that of the SAG and RES groups at 3 months of age. *Desulfovibrio* population was increased by sanguinarine and resveratrol, whereas methanogenic archaea population was decreased by resveratrol. No difference was observed in alpha and beta measures among all groups at 6 months of age. In summary, dietary sanguinarine or resveratrol affected rumen fermentation parameters and bacterial community in calves during 3-5 months of age. No effects of plant extract on rumen environment was detected at 6 and 9 months of age.

Key words: sanguinarine; resveratrol; rumen fermentation; bacterial community; calves

1. Introduction

Public concerns of the use of antibiotics in livestock production has increased due to the emergence of antibiotic resistant that may threaten human health (Benchaar et al., 2008). Consequently, the development of alternative strategies has attracted attention from many researchers. Plant extracts are among the most promising alternatives to antibiotics owing to their safety and extensive biological activities, and can be used in animal feeds (Hart et al., 2008).

Sanguinarine (SAG) is a quaternary benzo[c]phenanthridine alkaloid derived from the roots of *Sanguinaria Canadensis* and other poppy-fumaria species, and in the seeds of *Argemone mexicana L.* (Shamma and Guinaudeau, 1986). Due to its quaternary nitrogen, polycyclic and planar structure, SAG can react with nucleophilic and anionic moieties of amino acids in various biomacromolecules, receptors, and enzymes (Schmeller et al., 1997). SAG possess a wide range of pharmacological effects, including anti-inflammatory and antimicrobial properties (Mahady and Beecher,1994; Lenfeld et al., 1981; Chaturvedi et al., 1997). Dietary SAG have been reported to promote growth, decrease inflammatory response and improve intestinal morphology in swine, poultry and aquatic species (Kantas et al., 2015; Gudev et al.,2004; Lee et al., 2015; Rawling et al., 2009; Zhang et al., 2019). Resveratrol (RES) is a natural polyphenol (3,5,4'-trihydroxy-*trans*-stilbene) normally derived from grapes, peanuts, and knotweeds (Athar et al., 2007; Oyenihi et al., 2016). RES has been proven to possess extensive biological benefits, including antioxidant, anti-inflammatory and antimicrobial activities (Athar et al., 2007; Taguri et al., 2006; Paulo et al., 2010). Recent research in our laboratory indicates that oral administration of RES affects rumen fermentation and decreases methanogenesis in ewes (Ma et al., 2015). Until now, the efficacy of these two plants has rarely been investigated in calves.

The gastrointestinal tract (GI) of a calf is supposed to be sterile and germ free right after birth, and then, microbes from the surrounding environment subsequently colonize the rumen until a very complex and diverse microbial population develops (Ziolecki and Briggs, 1961). Nutritional interventions in early life of calves may promote different microbial populations establishing in the rumen of the young animal (Yáñez-Ruiz et al., 2015; Abecia et al., 2013). Both SAG and RES can inhibit a wide range of bacteria (Schmeller et al.,1997; Taguri et al., 2006; Paulo et al., 2010), but whether they can facilitate or prevent the colonization of microbial populations remains to be determined. Here, we investigated the effects of supplementing feed with SAG or RES on the environment of the developing rumen of young calves, and we followed them up to 9 months of age to determine the persistence of any effects.

2. Materials and methods

2.1Animals and Treatment, and Management

Fifty-four female Holstein calves at Yin Xiang dairy farm (Shandong province, China) were collected and randomly assigned to three treatment groups at 7 days of age. Calves were fed a basal diet of milk replacer alone (MR group), basal diet supplemented with 0.05 mg/kg BW SAG (>99 g/100g purity; extracted from *Macleaya cordata*; Micolta Bioresource., China; SAG group) or basal diet supplemented with 4 mg/kg BW RES (>99 g/100g purity; extracted from giant knotweed rhizome; Micolta Bioresource, China) (RES group). Feeding approximately 0.07-0.2 mg/kg BW of SAG to steers affected rumen fermentation parameters (Aguilar-Hernández, et al., 2016), thus we adopted a dose of 0.05 mg/kg BW of SAG to conduct this trial. A recent study in our lab demonstrated that feeding 4 mg/kg BW of RES to ewes affected rumen fermentation parameters (Ma et al., 2015), thus we adopted a dose of 4 mg/kg BW of RES. A milk replacer was reconstituted to an emulsion (125 g/L) by cooling boiled water. Calves were fed twice daily using plastic buckets. The feeding amount of MR emulsion was 5.0, 5.4, 6.2, and 7.3 kg during days 7-14, 14-28, 28-42, and 42-60, respectively. A starter feed and clean water were provided *ad libitum* beginning at 14 days. Weaning was conducted gradually from 61-64 days. The feeding amount of MR emulsion was 5.5, 3.7,1.8, and 0 kg on day 61, 62, 63, and 64, respectively. Forage was supplemented *ad libitum* beginning at 64 days. Plant extracts were added to MR at 7-63 days and mixed in starter feed at 64-180 days. No plant extract was used at 181-270 days. Plant extracts were firstly dissolved in approximate 2 kg of MR emulsion. And then they were thoroughly mixed with the rest of MR emulsion for 180 s by an agitator of a mobile calf feeding equipment (120×80×148 cm) (MilchMobil, Foerster-Technik, Germany). Calves were raised individually in calf hutches during days 7-63, then grouped by treatment in barns for days 64-180, and finally grouped as a single experimental cohort for days 181-270 (n =18 per each group). The experiment was conducted from days 7 to 270. MR and concentrate feeds were sampled biweekly, and nutrients were analyzed according to AOAC(1995) protocols. MR was purchased from the Beijing Precision Animal Nutrition Center, Beijing, China, and its patent number is CN02128844.5. The ingredient composition and nutrient value of basal diet are presented in Table 6. The experimental protocol was approved by the Animal Ethics Committee of the Chinese Academy of Agricultural Sciences (Beijing, China).

Table 6. Composition and chemical analysis of milk replacer and concentrate feeds (dry matter basis)

Ingredients (g/kg)	Concentrate feed 0-3 Month	Concentrate feed 4-6 Month

Corn[a]	556.5	565.0
Soybean Meal[a]	262.0	231.5
Extruded Soybean	70	0
Wheat bran	39	100
DDGS	30	60
Calcium Carbonate	22.5	23.0
Dicalcium Phosphate	6	5.5
Salt	4	5
Premix[b]	10	10

Nutrients (g/kg)	Milk replacer[c]	Concentrate feed 0-3 Month	Concentrate feed 4-6 Month
Dry Matter	946.8	879.4	877.7
Crude Protein	229.3	200.0	180.0
Crude Fat	160.2	38.6	32.1
Neutral Detergent Fiber	50.7	97.9	114.1
Acid Detergent Fiber	15.2	37.7	39.5
Crude Ash	43	69.4	69.9
Calcium	9	10.0	10.0
Total Phosphate	4.9	4.5	4.5
Salt	3.5	4.7	5.8

[a] The screen size of grinding mill is 2.5 mm for corn and 3.0 mm for soybean meal.

[b] provided per kg of concentrate feed: 10 000 IU vitamin A, 1 500 IU vitamin D, 60 IU vitamin E, 1.5 mg vitamin B1, 8.2 mg vitamin B2, 2 mg vitamin B6, 3.5 mg vitamin K, 1 mg folic acid, 0.1 mg biotin,49.5 mg niacin, 60 mg D-pantothenic acid, 10.2 mg Cu, 20 mg Fe, 140 mg Zn, 2 mg Mn, 2 mg I, and 0.44 mg Se.

[c] The patent number of milk replacer is CN02128844.5.

2.2Measurements

2.2.1 Growth

All calves were weighted prior to the morning feeding at the beginning of the trial (7 days of age), and 2 and 6 months of age. The starter feed intake was weighted on the 7 consecutive days prior to weaning at 60 d old (n =18 per each group).

2.2.2 Sampling

Twenty-four calves were randomly chosen for the ruminal liquid sampling (n = 8 per each group). Rumen fluid was collected at 2-3 h after morning feeding at 1, 2, 3, 4, 5, 6 and 9 months of age using an oral

stomach tubing technique. A stainless steel pipe was inserted into the rumen and then a 150 ml syringe was used to aspirate rumen fluids. The first 100 mL of fluid was discarded to avoid saliva contamination. Samples were filtered with double-layer gauze and collected in two vacuum tubes. One tube, containing 2 mL of sample, was placed immediately in liquid nitrogen and used for bacterial determination. The other tube, containing 15 mL of sample, was kept at –20°C and used for analysis of VFA and ammonia nitrogen.

2.2.3 Rumen fermentation parameters

Ruminal pH was determined immediately after sampling using a digital pH meter (Basic pH meter PB-20, Startorius AG, Germany). The concentration of VFA was determined as described previously (Zhang et al., 2016). Briefly, 1 mL rumen fluid filtrate was added to 0.2 mL of metaphosphoric acid solution (250 g/L) containing 2 g/L 2-ethyl butyrate, mixed overnight, and analyzed by gas chromatography (SP-3420, Beijing Analytical Instrument Factory, China). The concentration of ammonia nitrogen (NH_3-N) was measured using the phenol hypochlorite colorimetric method as described (Broderick and Kang, 1980).

2.2.4 DNA extraction and preparation of amplicons for high-throughput sequencing.

Bacterial DNA was extracted from1 mL of ruminal fluid using the QIAamp Fast DNA Stool Mini kit (Qiagen, Germany). The V4 region of the 16S rRNA gene was targeted for amplification using modified 515F/806R primers (515F: 5′-GTG CCA GCM GCC GCG GTA A-3′; 806R 5′-XXX XXX GGA CTA CHV GGG TWT CTA AT-3′). PCR mixtures (30 μL) contained 15 μL of 2× Phusion High-Fidelity PCR Master Mix (New England Biolabs, USA), 10 μL of 1 ng/μL DNA, 3 μL of 2 μM forward and reverse primers, and 2 μLHPLC-grade water. The amplification program consisted of one cycle of 98°C for 1 min, 30 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 30 s, and a final cycle of 72°C for 5 min. PCR products were purified using the GeneJET Gel Extraction kit (Thermo Scientific, Germany). Illumina paired-end sequencing libraries were constructed using the NEBNext DNA Sample Preparation kit (New England Biolabs, USA). DNA quality was checked using the Agilent 2100 Bioanalyzer, and DNA was quantified with the Qubit 2.0 Fluorometer (Invitrogen, USA). Sequencing of bacterial 16S rRNA was performed on the Illumina HiSeq2500 platform by Novogene Bioinformatics Technology Company (China).

2.2.5 Bioinformatic analysis

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer. Fast Length Adjustment of SHort Reads (known as FLASH, v1.2.7) were used to merge paired-end Illumina fastq files based on overlapping regions within paired-end reads. Low-quality raw tags were filtered through a quality-control pipeline using Quantitative Insights Into Microbial

Ecology (QIIME,v1.7.0) (Bokulich et al., 2013). The algorithm UCHIME was used to identify and remove chimeric sequences using the Gold database (Hass et al., 2011). Sequences were then assigned to OTUs at the 97% similarity level using UPARSE (v7.0.1001) (Edgar, 2013). Representative OTUs were assigned to bacterial taxonomies using the SILVA SSUrRNA database and RDP classifier via QIIME (Wang et al., 2007). Alpha diversity as indicated by observed species, Shannon index, Simpson index, Chao1, and abundance-based coverage estimator (ACE) were analyzed with QIIME (v1.7.0) and displayed with R software (v2.15.3). Principal coordinate analysis (PCoA) based on unweighted UniFrac distance was conducted to evaluate differences in community structure among experimental groups by R package vegan.

2.2.6 Droplet digital PCR

Populations of *Desulfovibrio* and methanogens were quantified using the QX200 Droplet Digital PCR System (Bio-Rad, USA) and the following primers: *Desulfovibrio*: DSV691-F(5′-CCGTAGATATCTGGAGGAACATCAG-3′) and DSV826-R (5′-ACATCTAGCATCCATCGTTTACAGC-3′) ; methanogenic archaea: qmcrA-F(5′-TTCGGTGGATCDCARAGRGC-3′) and qmcrA-R (5′-GBARGTCGWAWCCGTAGAATCC-3′). Briefly, ddPCR mixtures contained 10 μL QX 200 ddPCR EvaGreen supermix (Bio-Rad), 2 μL DNA, 0.2 μL forward primers, 0.2 μL reverse primers and 7.6 μL double-distilled water. Reaction mixtures (20 μL) were added to the middle wells of a DG8 cartridge, and 70 μL droplet generator oil was added to the bottom wells. Droplets were generated in the top wells using the Droplet Generator (Bio-Rad QX-200) and transferred to a 96-well PCR plate, which was heated-sealed with a foil seal (PX1 PCR Plate Sealer, Bio-Rad) and placed in a T100 Thermal Cycler (Bio-Rad). The cycling program was: one cycle of 95°C for 5min, 40 cycles of 95°C for 30s and 60°C for 1 min, and one cycle of 4°C for 5 min and 90°C for 5 min, with final hold at 4 °C. After PCR amplification, droplets were analyzed in the QX200 Droplet Reader (Bio-Rad, USA), and the copy number of PCR targets was calculated using QuantaSoft software (Bio-Rad, USA).

2.3Statistical analysis

Data for rumen fermentation parameters, alpha-diversity indices, and quantification of *Desulfovibrio* and methanogenic archaea were analyzed using the general linear model of SAS (SAS Version 8.01, SAS Institute, Inc., USA). The copy numbers of bacteria were $\log_{10}$ transformed before statistical analysis. Tukey's HSD test was conducted when a significant difference was detected among means. Alpha-diversity indices were analyzed by Wilcoxon test. Permutational MANOVA test was performed in R in the vegan package with the Adonis function. Different bacterial taxa between MR3 and SAG3 or MR3 and RES at genus level were analyzed by *t* test; only those with

relative abundance > 0.1% in at least one sample were visualized using R software. A value of $P < 0.05$ was considered statistically significant.

The non-parametric Spearman rank correlation was implemented using the PROC CORR procedure of SAS to identify correlations between rumen fermentation parameters and bacterial abundance. Only bacterial genera with relative abundance > 0.5% in at least one sample were included in the correlation analysis. Correlations with a threshold of statistical significance at P <0.0001 were visualized via heatmap generated using the corrplot package of R software.

3. Results

3.1Growth

Oral administration of SAG or RES had no effect on starter feed intake of calves during 54- 60 d. The average daily gain (ADG) of calves during 7 d-2 m, 2 m-6 m and 7 d-6 m were not affected by dietary plant extracts (Table 7).

Table 7. Effect of dietary plant extracts on growth performance of calves.

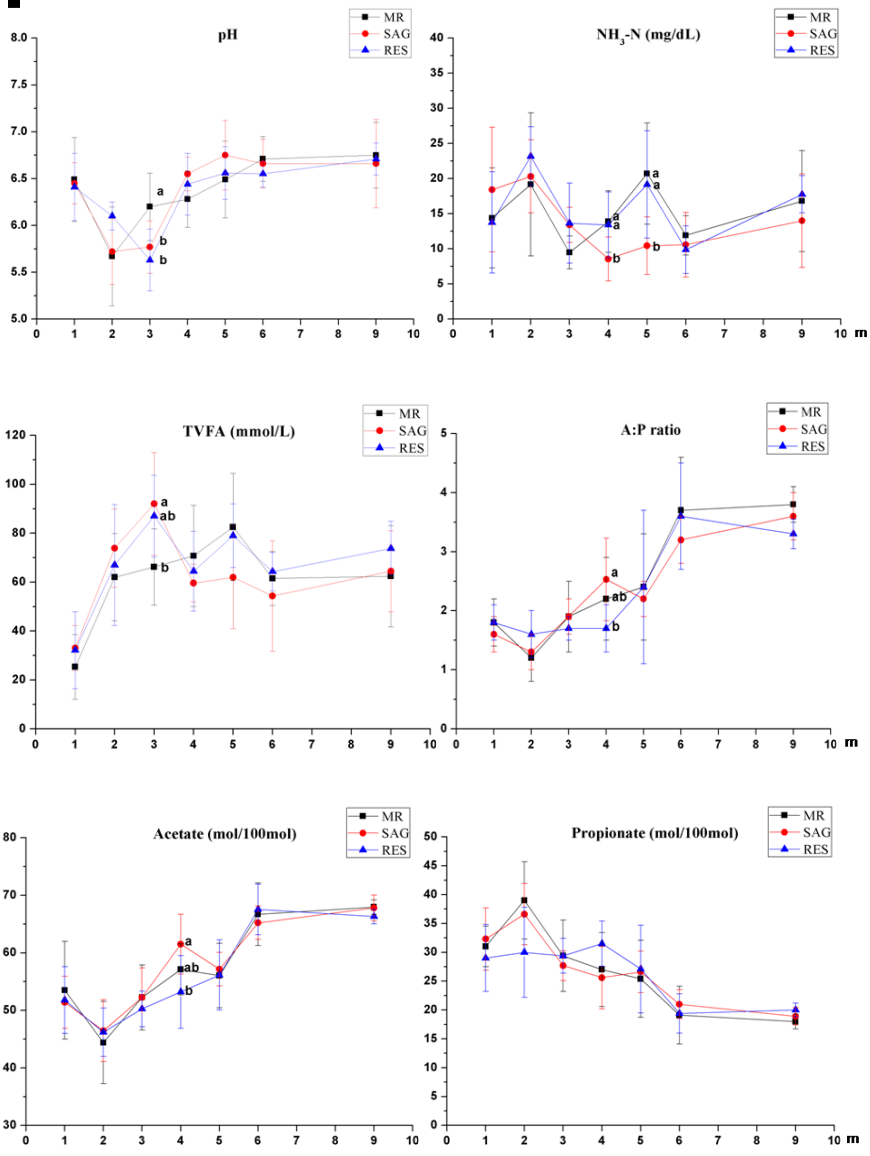
Items	MR	SAG	RES	SEM	*P* value
Initial BW (kg)	42.0	42.5	42.2	0.844	0.913
Starter feed intake (g)					
54 d~60 d	811.5	1029.6	910.3	87.446	0.579
ADG (g)					
7 d~2 m	504.3	527.1	519.1	29.929	0.873
2 m~6 m	942.7	998.5	1030.9	31.704	0.287
7 d~6 m	824.0	863.0	889.9	25.720	0.345

MR: a basal diet; SAG: the basal diet supplemented with 0.05 mg/kg BW sanguinarine; RES: the basal diet supplemented with 4 mg/kg BW resveratrol. Initial BW: initial body weight; ADG: average daily gain;

3.2Rumen fermentation parameters

No differences were observed in rumen fermentation parameters among groups at 1, 2, 6, and 9 months of age (Figure 13, numerical values refer to Table S5). At 3-month of age, ruminal pH was lower in the SAG and RES groups than in the MR group (P = 0.015). The concentration of total volatile fatty acids (TVFA) was greater in the SAG group than in the MR group (P = 0.048), while intermediate values were observed in RES group. At 4 months, the NH_3-N concentration and the molar proportion of butyrate were lower in the SAG group than in the other two groups (P = 0.029; P = 0.024, respectively). The molar proportion of acetate and the ratio of acetate to propionate (A:P ratio) were lower in the RES group than in SAG group (P = 0.023; P = 0.044, respectively), while intermediate values were observed in MR group.

The molar proportion of valerate was greater in the RES group than in the other groups (P = 0.038). At 5 months, the NH_3-N concentration was lower in the SAG group than in the other groups (P = 0.016). The molar proportion of valerate was lower in the RES and SAG groups than in the MR group (P = 0.029). No differences were observed in the molar proportions of propionate or isobutyrate.



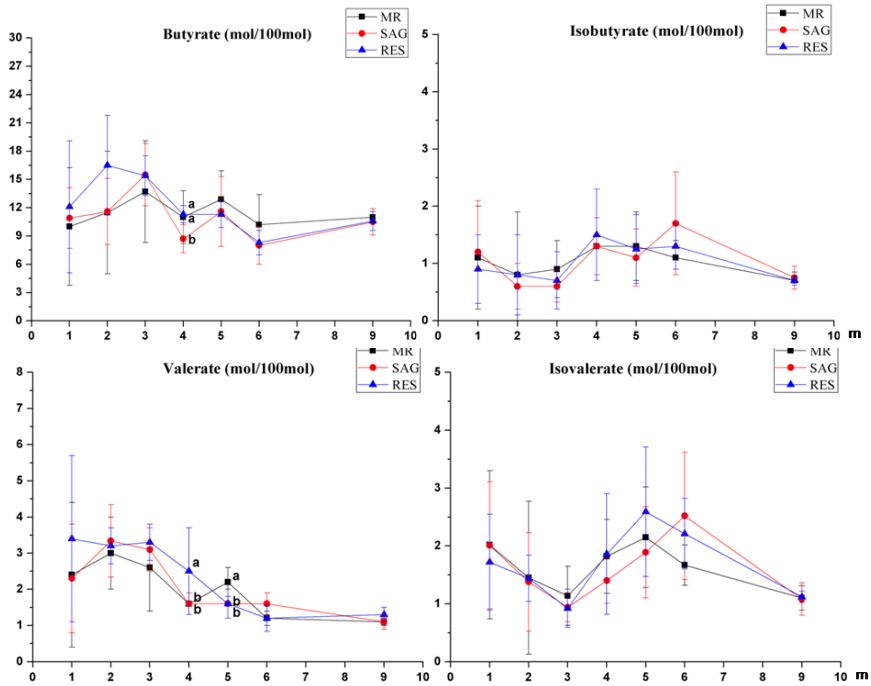


Figure 13. Rumen fermentation parameters.
MR: a basal diet; SAG: the basal diet supplemented with 0.05 mg/kg BW sanguinarine; RES: the basal diet supplemented with 4 mg/kg BW resveratrol. NH_3-N: ammonia nitrogen; TVFA: total violate fatty acids; Acetate, propionate, butyrate, isobutyrate, and valerate are presented as molar proportions relative to TVFA; A:P ratio: the ratio of acetate to propionate. [ab]Mean values at the same sampling month with different superscripts are different at $P < 0.05$.

Table S5. Rumen fermentation parameters.

1 Month	MR	SAG	RES	SEM	P value
pH	6.5	6.4	6.4	0.126	0.910
NH_3-N (mg/dL)	14.4	18.4	13.8	2.771	0.466
TVFA (mM)	25.4	33.0	32.2	4.592	0.481
Acetate (mol/100mol)	53.5	51.4	51.8	2.310	0.791
Propionate (mol/100mol)	31.0	32.3	29.2	1.784	0.461
Butyrate (mol/100mol)	10.0	10.8	12.1	2.027	0.771

Isobutyrate (mol/100mol)	1.1	1.2	0.9	0.289	0.840
Valerate (mol/100mol)	2.4	2.2	3.3	0.362	0.501
Isovalerate (mol/100mol)	2.0	2.0	1.7	0.385	0.825
A:P ratio	1.8	1.6	1.8	0.137	0.604

2 Months	MR	SAG	RES	SEM	P value
pH	5.7	5.7	6.1	0.136	0.133
NH_3-N (mg/dL)	19.2	20.3	23.2	2.629	0.656
TVFA (mM)	62.1	73.9	67.0	6.894	0.587
Acetate (mol/100mol)	44.4	46.5	46.2	2.084	0.791
Propionate (mol/100mol)	38.9	36.6	30.1	2.302	0.133
Butyrate (mol/100mol)	11.5	11.6	16.5	1.878	0.238
Isobutyrate (mol/100mol)	0.8	0.6	0.8	0.289	0.864
Valerate (mol/100mol)	3.0	3.3	3.2	0.297	0.983
Isovalerate (mol/100mol)	1.4	1.4	1.4	0.350	0.992
A:P ratio	1.2	1.3	1.8	0.161	0.122

3 Months	MR	SAG	RES	SEM	P value
pH	6.2^{a}	5.8^{b}	5.6^{b}	0.129	0.015
NH_3-N (mg/dL)	9.5	13.4	13.7	1.481	0.110
TVFA (mM)	66.2^{b}	91.9^{a}	87.2^{ab}	7.196	0.048
Acetate (mol/100mol)	52.3	52.2	50.3	1.919	0.704
Propionate (mol/100mol)	29.4	27.7	29.4	1.727	0.721
Butyrate (mol/100mol)	13.7	15.5	15.4	1.551	0.670
Isobutyrate (mol/100mol)	0.9	0.6	0.7	0.182	0.512
Valerate (mol/100mol)	2.6	3.1	3.3	0.344	0.368
Isovalerate (mol/100mol)	1.1	0.9	0.9	0.145	0.512
A:P ratio	1.9	1.9	1.7	0.165	0.689

4 Months	MR	SAG	RES	SEM	P value
pH	6.3	6.6	6.4	0.096	0.148

NH_3-N (mg/dL)	13.9[a]	8.6[b]	13.4[a]	1.437	0.029
TVFA (mM)	70.7	59.6	64.5	5.593	0.409
Acetate (mol/100mol)	57.1[ab]	61.5[a]	53.2[b]	1.874	0.023
Propionate (mol/100mol)	27.1	25.6	31.5	1.845	0.114
Butyrate (mol/100mol)	11.0[a]	8.7[b]	11.3[a]	0.648	0.024
Isobutyrate (mol/100mol)	1.3	1.3	1.5	0.240	0.774
Valerate (mol/100mol)	1.6[b]	1.6[b]	2.5[a]	0.270	0.038
Isovalerate (mol/100mol)	1.8	1.4	1.9	0.267	0.449
A:P ratio	2.2[ab]	2.5[a]	1.7[b]	0.209	0.044

5 Months	MR	SAG	RES	SEM	P value
pH	6.5	6.8	6.6	0.127	0.340
NH_3-N (mg/dL)	20.7[a]	10.4[b]	19.2[a]	2.558	0.016
TVFA (mM)	82.5	62.2	79.0	6.794	0.101
Acetate (mol/100mol)	56.0	57.2	56.1	1.797	0.887
Propionate (mol/100mol)	25.4	26.6	27.1	2.198	0.864
Butyrate (mol/100mol)	12.9	11.6	11.3	1.012	0.505
Isobutyrate (mol/100mol)	1.3	1.1	1.2	0.203	0.714
Valerate (mol/100mol)	2.2[a]	1.6[b]	1.6[b]	0.152	0.029
Isovalerate (mol/100mol)	2.1	1.9	2.6	0.333	0.336
A:P ratio	2.4	2.2	2.4	0.326	0.882

6 Months	MR	SAG	RES	SEM	P value
pH	6.7	6.7	6.6	0.082	0.352
NH_3-N (mg/dL)	11.9	10.6	9.9	1.383	0.565
TVFA (mM)	61.5	54.3	64.3	5.654	0.437
Acetate (mol/100mol)	66.7	65.2	67.5	1.656	0.598
Propionate (mol/100mol)	19.1	21.0	19.4	1.429	0.633
Butyrate (mol/100mol)	10.2	8.0	8.3	0.851	0.172
Isobutyrate (mol/100mol)	1.1	1.7	1.3	0.224	0.146
Valerate (mol/100mol)	1.2	1.6	1.3	0.115	0.063

Isovalerate (mol/100mol)	1.7	2.5	2.2	0.287	0.135
A:P ratio	3.7	3.2	3.6	0.295	0.389

MR: a basal diet; SAG: the basal diet supplemented with 0.05 mg/kg BW sanguinarine; RES: the basal diet supplemented with 4 mg/kg BW resveratrol. NH_3-N: ammonia nitrogen; TVFA: total violate fatty acids; Acetate, propionate, butyrate, isobutyrate, and valerate are presented as molar proportions relative to TVFA. A:P ratio: the ratio of acetate to propionate.

[ab]Mean values at the same sampling month with different superscripts are different.

3.3Rumen bacterial community

A total of 2,388,941 high-quality DNA sequences were obtained from 46 samples of rumen liquid (51,934±2,675 sequences/sample, mean ± SEM). High-quality reads were clustered using >97% sequence identity into 2329 microbial operational taxonomic units (OTUs). Rarefaction curves demonstrated sufficient OTU coverage to accurately describe the bacterial composition (Figure S3). A total of 24 phyla were identified, with *Bacteroidetes*, *Firmicutes* and *Proteobacteria* being the predominant phyla. The average abundance of phylum *Bacteroidetes* in the SAG and RES groups was reduced in 3- and 6-month-old calves (Figure 14).

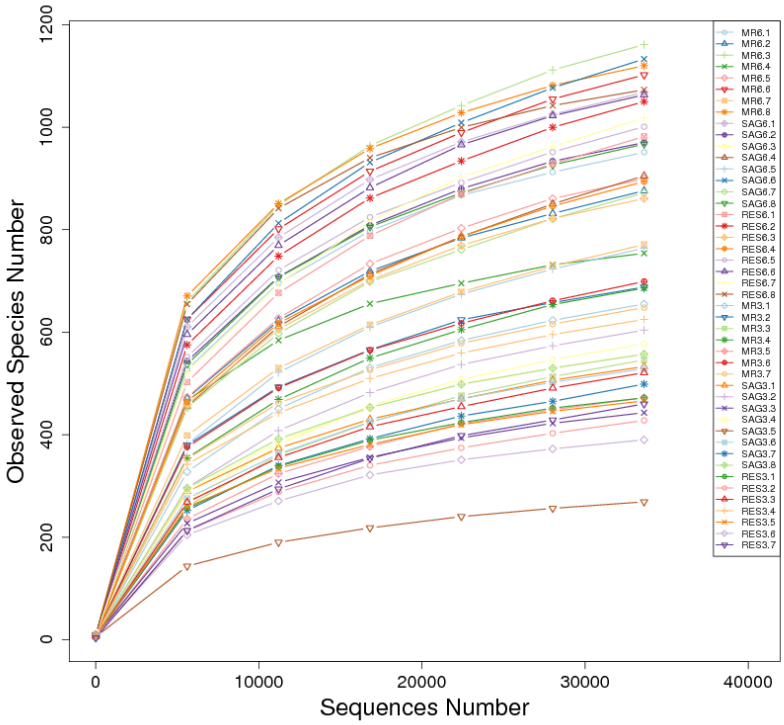


Figure S3. Rarefaction curves
MR3.1-3.7, SAG3.1-3.8, RES3.1-3.8: Rumen samples at 3 months of age in the basal diet (MR3.1-3.7), or supplemented with 0.05 mg/kg BW sanguinarine (SAG3.1-3.8) or 4 mg/kg BW resveratrol (RES3.1-3.8). MR6.1-6.8, SAG6.1-6.8, RES6.1-6.8: Rumen samples at 6 months of age in the basal diet (MR6.1-6.8), or supplemented with 0.05 mg/kg BW sanguinarine (SAG6.1-6.8) or 4 mg/kg BW resveratrol (RES6.1-6.8).

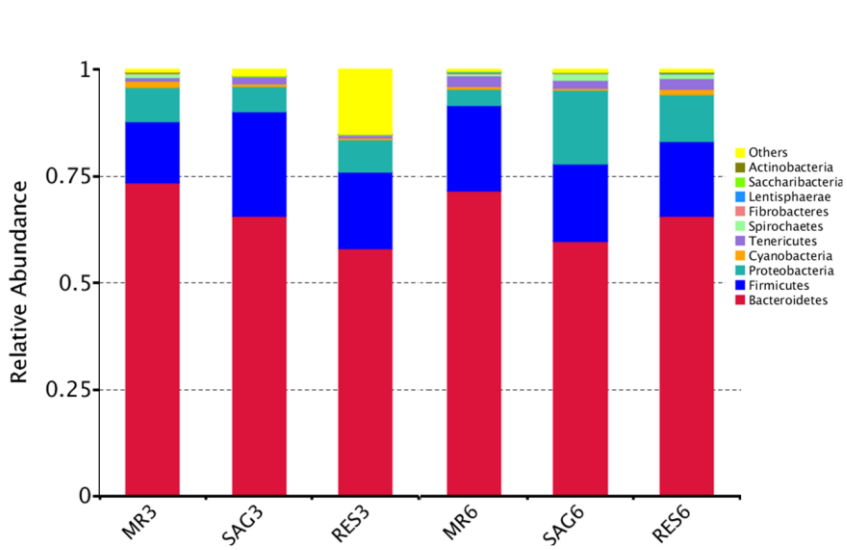


Figure 14. Average relative abundance of rumen bacteria stratified by treatment and age at the phylum level. The top 15 abundant phylum were presented.
MR3, SAG3, RES3: Rumen samples at 3 months of age in the basal diet (MR3), or supplemented with 0.05 mg/kg BW sanguinarine (SAG3) or 4 mg/kg BW resveratrol (RES3). MR6, SAG6, RES6: Rumen samples at 6 months of age in the basal diet (MR6), or supplemented with 0.05 mg/kg BW sanguinarine (SAG6) or 4 mg/kg BW resveratrol (RES6).

Among 3-month-old calves, the observed species, Chao1, and ACE values were greater in the MR group than in the other groups (P = 0.0120; P = 0.0284; P = 0.0248, respectively) (Table 8). No difference in alpha measures was observed for 6-month-old calves.

Table 8. Alpha-diversity measures at 3 months of age stratified by treatment.

Alpha diversity	MR3	SAG3	RES3	SEM	*P* value
Observed species	627.7[a]	499.0[b]	480.3[b]	31.864	0.0120
Shannon	5.04	4.81	4.29	0.299	0.2647
Simpson	0.90	0.91	0.82	0.0391	0.2630
Chao1	768.5[a]	606.1[b]	596.9[b]	43.872	0.0284

ACE	782.5[a]	627.9[b]	612.8[b]	41.675	0.0248

[ab]Mean values at the same sampling month with different superscripts are different.

Beta-diversity measures of principal coordinate analysis (PCoA) based on unweighted UniFrac distance revealed a clear diversification of bacterial community between 3 months and 6 months (Figure 15). An analysis of similarity randomization test (ANOSIM) confirmed significant differences between MR3 and MR6 (R = 0.707, *P* = 0.003), SAG3 and SAG6 (R = 0.6777, *P* = 0.001), or RES3 and RES6 (R = 0.6939, *P* = 0.001). The community structure of bacteria in the MR3 group was distinct from that of the SAG3 (ANOSIM, R = 0.266, *P* = 0.013) and RES3 groups (ANOSIM, R = 0.198, *P* = 0.021).

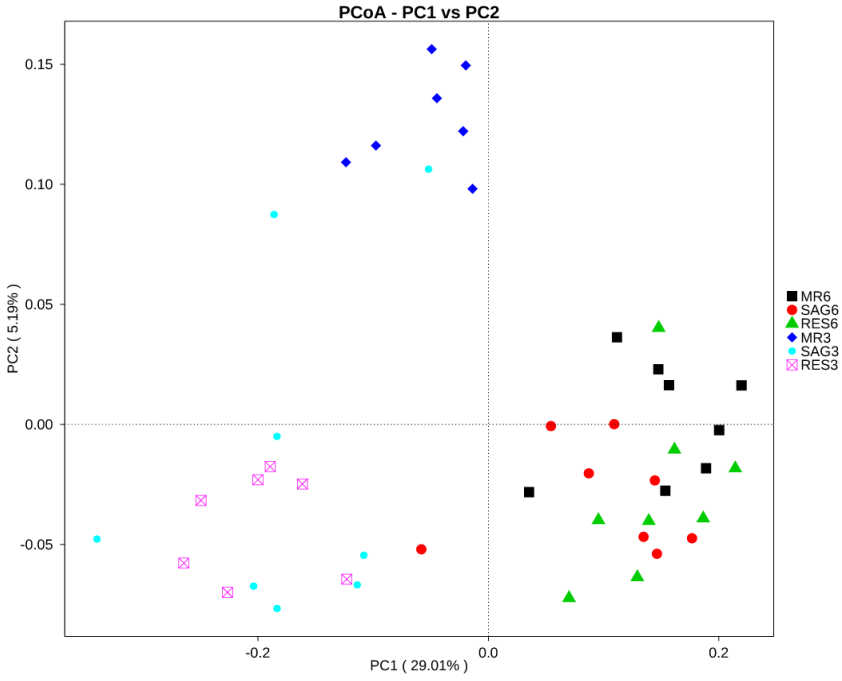


Figure 15. Beta-diversity measures of the rumen bacterial community by principal coordinate analysis based on unweighted UniFrac distance. MR3, SAG3, RES3: Rumen samples at 3 months of age in the basal diet (MR3), or supplemented with 0.05 mg/kg BW sanguinarine (SAG3) or 4 mg/kg BW resveratrol (RES3). MR6, SAG6, RES6: Rumen samples at 6 months of age in the basal diet (MR6), or supplemented with 0.05 mg/kg BW sanguinarine (SAG6) or 4 mg/kg BW resveratrol (RES6). Permutational MANOVA test was performed to rank the distances calculated using the community data. Significance were detected between MR3 vs. MR6 (R = 0.707, *P* = 0.003), SAG3 vs. SAG6 (R = 0.6777, *P* = 0.001), RES3 vs.

RES6 (R = 0.6939, *P* = 0.001), MR3 vs. SAG3 (R = 0.266, *P* = 0.013), MR3 vs. RES3 (R = 0.198, P = 0.021)

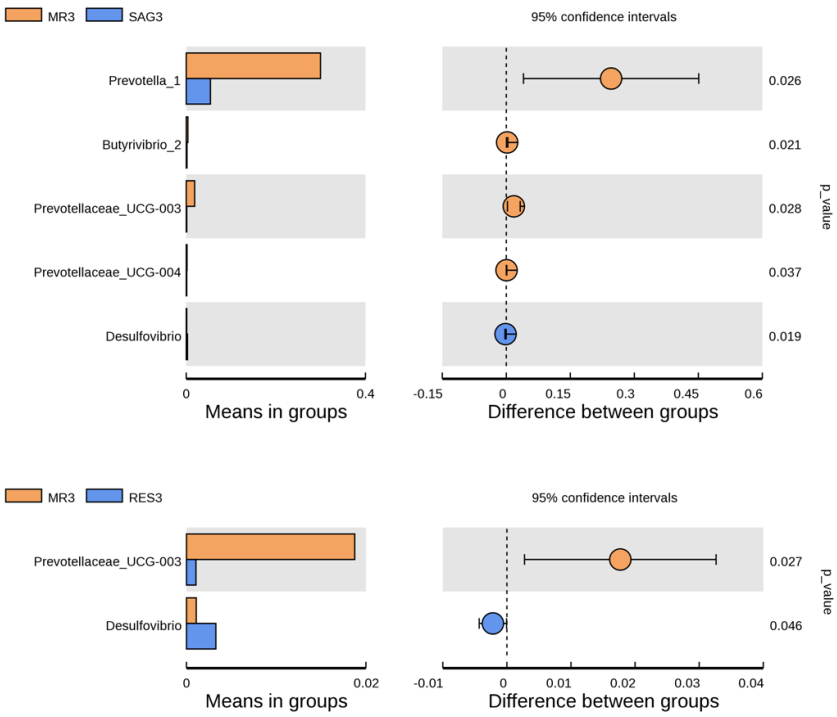


Figure 16. Statistical analysis of different bacterial taxa at the genus level by *t* test;only those with relative abundance > 0.1% in at least one sample were visualized using R software.
MR3, SAG3, RES3: Rumen samples at 3 months of age in the basal diet (MR3), or supplemented with 0.05 mg/kg BW sanguinarine (SAG3) or 4 mg/kg BW resveratrol (RES3).

Statistical analysis revealed that the genera *Prevotella 1*, *Butyrivibrio 2*, *Prevotellaceae UCG-003* and *Prevotellaceae UCG-004* were more abundant in MR3 than in SAG3 (*P* = 0.026; *P* = 0.021; *P* = 0.028; *P* = 0.037, respectively), whereas the genus *Desulfovibrio* was more abundant in SAG3 than in MR3 (*P* = 0.019). The genus *Prevotellaceae UCG-003* was more abundant, and *Desulfovibrio* was less abundant, in MR3 than in RES3 (*P* = 0.027; *P* = 0.046, respectively;

3.4Correlation analysis

Correlations between rumen fermentation parameters and bacterial abundance at the genus level are presented in Figure 17. Ruminal pH was positively correlated with *Rikenellaceae RC9 gut group, Butyrivibrio 2,* unidentified Bacteroidales RF16 group, *Prevotellaceae UCG-003, Treponema 2, Anaeroplasma, Thalassospira, Prevotellaceae Ga6A1 group, Ruminococcaceae NK4A214 group, Saccharofermentans, Prevotellaceae UCG-001, Prevotellaceae UCG-004, Coprococcus 2, Erysipelotrichaceae UCG-004, probable genus 10,* and *Ruminococcaceae UCG-010,* and negatively correlated with *Prevotella 7, Dialister,* and unidentified *Prevotellaceae.*

TVFA was negatively related to *Treponema 2, Thalassospira, Prevotellaceae Ga6A1 group, Ruminococcaceae NK4A214 group* and *Coprococcus 2*. Acetate was positively correlated with *Rikenellaceae RC9 gut group, Butyrivibrio 2,* unidentified *Bacteroidales RF16 group, Treponema 2, Ruminococcaceae UCG-002, Anaeroplasma, Prevotellaceae Ga6A1 group, Ruminococcaceae NK4A214 group, Christensenellaceae R-7 group, Saccharofermentans, Prevotellaceae UCG-001, Prevotellaceae UCG-004, Coprococcus 2, Pseudobutyrivibrio, Erysipelotrichaceae UCG-004, probable genus 10, Ruminococcaceae UCG-010,* and negatively correlated with *Prevotella 7, Dialister,* and unidentified *Prevotellaceae.* In contrast, the opposite correlations were observed between propionate and these bacteria, with the exception of *Lachnoclostridium 12* and *Prevotellaceae UCG-004,* which showed no correlation. Correlations between the ratio of acetate to propionate and these bacteria were similar to the acetate correlations, except for *Prevotellaceae UCG-004,* which showed no correlation. Butyrate was negatively correlated with *Anaeroplasma, Saccharofermentans,* and *Ruminobacter*. Valerate was positively correlated with *Prevotella 7, Acidaminococcus, Dialister, Faecalibacterium,* and unidentified *Prevotellaceae,* and negatively correlated with *Rikenellaceae RC9 gut group, Butyrivibrio 2, Prevotellaceae UCG-003, Treponema 2, Anaeroplasma, Thalassospira, Prevotellaceae Ga6A1 group, Ruminococcaceae NK4A214 group, Christensenellaceae R-7 group, Saccharofermentans, Prevotellaceae UCG-001, Prevotellaceae UCG-004, Coprococcus 2, Pseudobutyrivibrio, Erysipelotrichaceae UCG-004, probable genus 10,* and *Ruminococcaceae UCG-010*. No significant correlation was detected between NH3 and the abundance of any bacterial species.

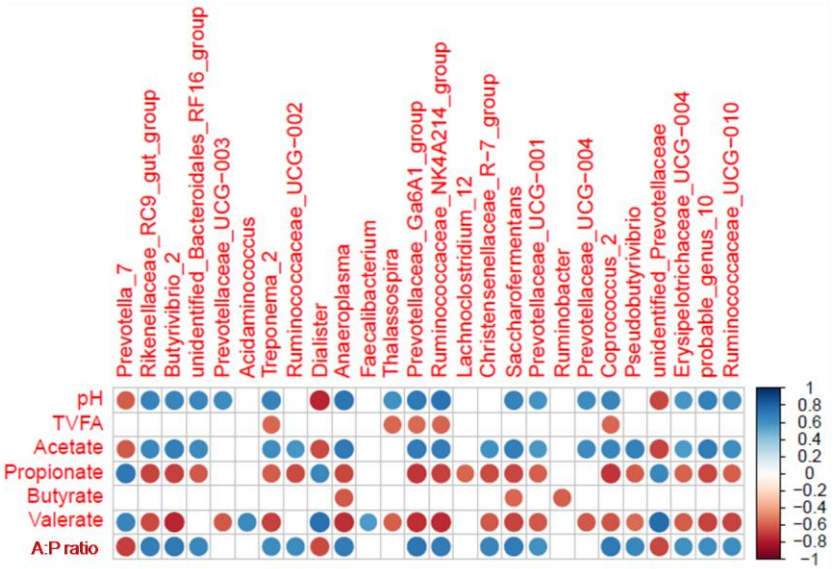
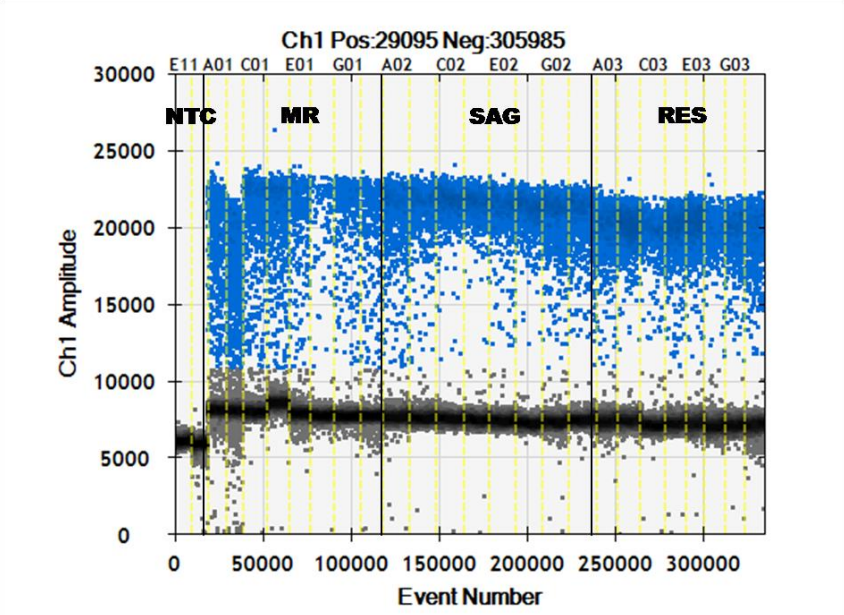


Figure 17. Spearman's rank correlations between rumen fermentation parameters and relative genus abundance.
TVFA: total violate fatty acids; Acetate, propionate, butyrate, isobutyrate, and valerate are presented as molar proportions relative to TVFA.
Blue and red represent positive and negative correlations, respectively; Only bacterial genera with relative abundance > 0.5% in at least one sample were included in the correlation analysis. Correlations with a threshold of statistical significance at P <0.0001 were visualized; color intensity reflects degree of correlation.

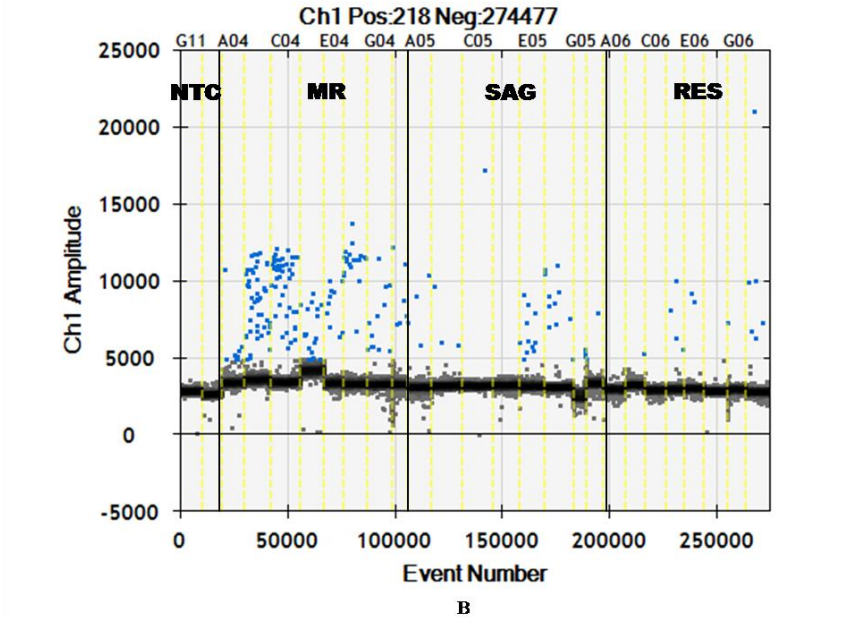
3.5Quantification of Desulfovibrio and methanogenic archaea

Amplitude analysis of positive and negative droplets by droplet digital PCR (ddPCR) is presented in Figure 18. For *Desulfovibrio,* 29,095 positive and 305,985 negative droplets were detected in 24 samples (n = 8) and two non-template controls (NTC). For methanogenic archaea, 218 positive and 274,477 negative droplets were identified in 24 samples (n = 8) and two NTC. The copy number of *Desulfovibrio* was greater in the SAG and RES groups than in the MR group (P = 0.0021; Table 9), and the copy number of methanogenic archaea was lower in the RES group than in the MR group (P = 0.0234).

No difference was observed in the copy number of methanogenic archaea between SAG and MR.



A



B

Figure 18. Amplitude analysis using droplet digital PCR for detection of *Desulfovibrio* (A) and methanogenic archaea (B)
MR: a basal diet; SAG: the basal diet supplemented with 0.05 mg/kg BW sanguinarine; RES: the basal diet supplemented with 4 mg/kg BW resveratrol.

Table 9. Copy number of *Desulfovibrio* and methanogenic archaea in Log_{10} (copies/mL fluid)

Items	MR	SAG	RES	SEM	*P* value
Desulfovibrio	6.16^{b}	6.58^{a}	6.68^{a}	0.0946	0.0021
Methanogenic archaea	4.72^{a}	3.56^{ab}	2.49^{b}	0.5243	0.0234

MR: a basal diet; SAG: the basal diet supplemented with 0.05 mg/kg BW sanguinarine; RES: the basal diet supplemented with 4 mg/kg BW resveratrol.
[ab]Mean values at the same sampling month with different superscripts are different.

4. Discussion

SAG, a benzophenanthridine alkaloid, has a wide spectrum of antimicrobial activities (Newton et al., 2002; Navarro et al., 1999), which were explained by increased membrane permeability (Schmeller et al.,1997) or inhibited proliferation of both gram-positive and gram-negative bacteria by perturbing FtsZ assembly dynamics in the Z ring (Beuria et al., 2005). It was reported that alkaloids had selective effects on microbial growth along the digestive tract (Cushnie et al., 2014). Dietary SAG has been reported to affect intestinal microflora in broiler chickens (Juskiewicz et al., 2011; Lee et al., 2015) and intestinal fermentation end-products in weanling piglets (Pellikaan et al., 2010). Until now, the effects of SAG on rumen fermentation and the rumen bacterial community in calves have not been documented.

Reduced bacterial richness and relative abundance of genus *Prevotella 1*, *Butyrivibrio 2*, *Prevotellaceae UCG-003*, and *Prevotellaceae UCG-004* in group SAG3 might be associated with the antimicrobial activities of SAG. Among these genera, *Butyrivibrio 2*, *Prevotellaceae UCG-003*, and *Prevotellaceae UCG-004* were positively correlated with ruminal pH, as indicated by a lower pH and higher TVFA concentration in SAG3. It has been reported that sulfate-reducing bacteria tolerate a wide pH range from 5.5 to 9.0 (Maree and Strydom, 1985). Dietary SAG increased the population of *Desulfovibrio* in digestive tract of koi fish (Zhang et al., 2019), which was in accordance with our results. *Desulfovibrio* accounts for the majority of dissimilatory sulfate-reducing activity in the rumen and can convert sulfate to sulfide. The final product, hydrogen sulfide (H_2S) is recognized as the third gaseous signal molecular (Wallace, 2010), which plays important roles

in mucosal defense in digestive system (Wang et al., 2002; Kimura et al., 2005). H_2S had protective effect on gastric ischemisa-reperfusion injury through decreasing oxidative stress and anti-inflammatory effect (Liu et al., 2010, Ju et al., 2013). SAG was known to possess anti-inflammatory properties and it had been used to treat various inflammatory diseases (Niu et al., 2012). Dietary supplementation with SAG decreases the concentration of serum amyloid A and haptoglobin in weaning piglets (Kantas et al., 2015). Rumen epithelium of ewes fed SAG had lower scores for cellular dropsical degeneration, parakeratosis, and neutrophil infiltration (Estrada-Angulo et al., 2016). Thus we speculated that anti-inflammatory activity of SAG might be possibly associated with the population of *Desulfovibrio* and its metabolite H_2S. However, inflammatory indices were not measured in this research, thus we can not affirm this hypothesis.

SAG has been reported to increase flow of non-ammonia N to small intestine, consequence of both decreased feed protein degradation and increased microbial protein synthesis in cattle and steers fed a high-energy diet (Plascencia and Zinn, 2014; Aguilar-Hernández, et al., 2016). It was in accordance with our results that SAG decreased the concentration of NH_3-N. It may be explained by decreased amino acid degradation from decarboxylation (Drsata et al., 1996). Additionally, SAG may exert antimicrobial effects on high ammonia-producing bacteria.

RES inhibits the proliferation of a wide range of bacteria in vitro (Taguri et al., 2006; Paulo et al., 2010). It may inhibit efflux pumps, thus increasing bacterial sensitivity (Tegos et al., 2002; Jung et al., 2009). Attempts to extend such findings to the manipulation of rumen function, using in vitro incubation, has illustrated that RES can decrease VFA concentrations and methane production (Becker and Van Wikselaar, 2011). A recent in vivo study demonstrated that feeding 0.25g/head per day of RES to ewes weighing 64-67.2 kg (4 mg/kg BW) increased the molar proportion of propionate, decreased the acteate/ propionate ratio, and decreased the molar proportion of butyrate (Ma et al., 2015). We found that feeding RES affected ruminal fermentation by reducing pH and causing fluctuation of the molar proportion of valerate.

RES has been found to increase the populations of *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Butyrivibrio fibrisolvens* in the rumen of ewes (Ma et al., 2015). Populations of methanogenic bacteria and protozoa, and the methane output scale for digestible dry matter, are both reduced by RES (Ma et al., 2015). In this trial, the population of *Desulfovibrio* was greater in the RES3 group. As reported previously, sulfate-reducing bacteria compete with methanogenic bacteria for substrates such as H_2 and acetate (Bryant et al., 1977; Oremland et al., 1982).

Thus, we speculated that *Desulfovibrio* might be a key genus associated with the population of methanogenic archaea, and therefore quantified

both of them. Digital droplet PCR (ddPCR) was proved to be a precise and reliable method for the absolute quantification of DNA copy number (Hindson et al., 2011), hence, it was newly employed to detect the copy number of *Desulfovibrio* and methanogenic archaea in rumen. The results verified an increase in *Desulfovibrio* and a decrease in methanogenic archaea due to dietary supplementation with RES. Similarly, previous research indicated hydrogenotrophic acetogens were gradually replaced by methanogenic archaea as the rumen develops (Gagen et al., 2012). Fonty et al. (2007) suggested that maintained inoculation with acetogens from early life might be a feasible option to decrease methane emissions in adult animals.

Walle et al.(2004) identified three metabolic pathways for RES in the human intestinal tract, including sulfate and glucuronic acid conjugation of the phenolic groups as well as hydrogenation of the aliphatic double bond. Rapid sulfate conjugation appears to be the rate-limiting step in the bioavailability of RES (Walle et al., 2004). Although metabolites of RES in the rumen remain unknown, both RES and its metabolites might play important roles in manipulating rumen bacteria. RES was shown to be capable of inhibiting growth of protozoa in both in vitro (Mallo et al., 2013) and in vivo (Ma et al., 2015) experiments, which was probably the other approach to reduce methane production. Our results illustrated that RES might be a promising additive for controlling methane production and thus deserves further investigation.

Previous studies have demonstrated that there is a strong host-microbiota specificity in the rumen (Weimer et al., 2010; Hook et al., 2009). The composition of the rumen microbial community is associated with the physiological condition of the host (Jami et al., 2014; McCann et al., 2014; Lima et al., 2015). Numerous attempts to modify rumen fermentation and microbiota have been reported, including transplanting digesta (Weimer et al., 2010), altering diet compositions (Machmüller et al., 1998; Popova et al., 2011), and exogenous addition of probiotics (Girard and Dawson, 1995; Guedes et al., 2008; Mwenya et al., 2004), organic acids (Foley et al., 2009) and ionophores (Hook et al., 2009; Odongo et al., 2007). However, the efficiency of these approaches has been limited because the rumen environment returns to its original state once the treatment is removed, reflecting the resilience of the ecosystem of the fully matured rumen (Weimer et al., 2010). As an alternative, such manipulations in the early life of ruminants - particularly during the period in which the microbiota is established - may provide a feasible window for programming the microbial ecosystem, giving rise to a more efficient and sustainable effect (Yáñez-Ruiz et al., 2015). Cellulolytic bacteria and other bacterial species important to rumen function can be detected as early as 1 day after birth (Jami et al., 2013). The presence of cellulolytic and methanogenic bacteria was observed in lambs at 3-4 days of age, and the population of these bacteria reached a level similar to that observed

in mature sheep within 7 days of age (Fonty et al., 1987). Thus, microbial programming by dietary intervention may be more effective during the 1st week of age. Based on our observations that supplementing plant extracts (SAG and RES) to calves did not yield long-term effects on rumen fermentation patterns or bacterial community. This can also reflect the strong adaptive capability of rumen bacteria. With increasing age, the anatomical volume of the rumen increases rapidly and the richness and diversity of rumen bacteria are enhanced dramatically, which might eliminate the impact of exogenous plant extracts. Additionally, the type or the level of plant extracts used in the present study may not be effective measures in creating changes among treatments. Feed intake was not measured after weaning and the effective quantity of plant extracts ingested by calves were not monitored, which was a limitation of this trial.

5. Conclusion

In conclusion, oral administration of SAG or RES to calves altered the rumen fermentation environment and bacterial community structure for a short period. The population of *Desulfovibrio* was increased by SAG and RES, whereas the population of methanogenic archaea was decreased by RES. However, feeding SAG or RES in early life did not cause lasting effects on the rumen environment, as both rumen fermentation parameters and the bacterial community were restored to their original state prior to 6 months of age.

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5

General discussion, Conclusion and Prospectivies

1. General discussion

Rumen is the most important digestive organ for cattle. As the rumen develops and colonized by microorganisms, a calf experiences a physiological transition from a pseudo-monogastric animal to a ruminant. During this period, the establishment of rumen microbiota is vulnerable to changes in the diet and external environment. This may provide an opportunity for rumen microbial programming.

Until now, research on microbial programming effect in ruminants is still limited. Alterations in the diet of maternal ewes and preweaning lambs or inoculation treatment have long-term effects on ruminal bacterial communities of lambs (Yáñez-Ruiz et al., 2010; De Barbieri et al., 2015). Our results reveal that early feeding regime of different liquid feed may lead to diverse microbial communities establishing in the rumen of calves. Feeding MR to calves produced a long-term effect on the rumen environment and microbiota. This adds a new evidence on microbial programming effect of nutrition in young ruminants. The experiment was conducted on the same calves from birth to 6 months of age. The results are more convinced than those data collected from separate trials on different farms.

PCR is a useful technology for detection and quantitation of specific nucleic acid sequences. The first generation of PCR realized quantitation using end-point analysis by gel electrophoresis. The second generation of real-time PCR enabled quantitation by monitoring the progression of amplification after each cycle using fluorescence probes. The third generation of digital droplet PCR (ddPCR) allows each sample partitioned into 15,000-20,000 droplets, which are subject to end-point PCR. The number of droplets with amplification product is then measured, allowing for an estimate of template density without the need for a standard curve (Hindson et al., 2011). ddPCR has been proved to be a precise and reliable method for the absolute quantification of DNA copy number, however, its application in rumen microbiota analysis is still limited. In this research, it was newly used to determine the population of bacteria in rumen, and the results agree well with 16 s rDNA sequencing data.

However, there are some problems that needed concern. Firstly, rumen fermentation profiles and microbiota are subject to the amount of leakage liquid in rumen. Nipple-fed calves may have different results from this research where calves were bucket-fed. Growth rate may also be different because liquid fermented in the rumen has less nutritional value for calves. Secondly, solid feed intake was not well determined. Starter feed intake was only measured for 7 days prior to weaning. The difference of feed intake was great among individuals, which might be associated with the inconsistence of leakage liquid in rumen or the use of straw bedding. Dry matter intake of concentrate feed and forage was not measured after the weaning. Thus, it is difficult to disassociate its effect on rumen environment from that of liquid feed. For example, the higher TVFA in calves fed MR are probably due to the greater starter

feed intake. However, due to lack of valid data on feed intake, it can also be attributed to the poor absorption of rumen epithelium caused by soy protein in MR. Thirdly, the bacterial community of rumen liquid was different from that of the solid fraction and that attached to the epithelium. Our results of ruminal liquids may not adequately represent the complexity of the rumen microbiome. Further surveys on the solid fraction of rumen content and epithelium-associated communities will provide additional information on microbiota.

1.1 The high level of CP, bioactive factors and antibiotics residuals in waste milk lead to a separated rumen bacterial community.

(1) The high protein content in waste milk may contribute to a greater concentration of isovalerate in rumen. Liu et al. (2014, 2016) reported that supplementation of isovalerate to calves and steers increased the population of *Butyrivibrio fibrisolvens* in rumen, which was in accordance with our result that the abundance of *Butyrivibrio fibrisolvens* was positively correlated with isovalerate in 2-month-old calves. The increased concentration of isovalerate may also be the reason to explain the increased ruminal bacterial richness. As a greater population of total bacteria was detected in the rumen of steers fed isovalerate (Liu et al., 2014). A similar result was observed that the number of OTUs in the rumen of calves fed pasteurized waste milk was higher than that in calves fed untreated whole milk (Deng et al., 2017).

Dietary supplementation of BCVFA can induce extraruminal effects that the level of GH in blood increased and that of insulin and NEFA decreased (Fieo et al., 1984; Towns and Cook, 1984; Liu et al., 2009). It mainly due to the existence of BCVFA receptors in ruminal and hepatic membranes, which can perturb the function of hormone-regulation system (Andries et al., 1987; Hamamdzic, 1989). A similar observation was found in our result that the level of GH and IGF-1 increased, whereas that of insulin and NEFA decreased. Thus, we believed it might be also associated with the greater concentration of isovalerate in the rumen of calves fed WM.

(2) Colostrums and transition milk are the main component of waste milk and contain a greater concentration of hormones and bioactive factors. This may also contribute to the increased concentration of GH and IGF-1 in the serum, which might play important roles in promoting the rumen epithelial cell proliferation (Baldwin, 1999; Zitnan et al., 2005). Moallem (2010) indicated that, compared with a MR diet, a WM diet had a positive effect on milk yield during the first lactation of the adults, which suggests a lactocine effect associated with WM.

(3) Antibiotics residuals in WM may lead to changes in rumen microbiota. Van Vleck et al. (2016) indicated that calves fed milk containing very low concentrations of antibiotics affected the

composition of microbial population in feces. We found the abundance of genus *Prevotella* decreased in calves fed WM, which was supported by a recent study (Li et al., 2019).

1.2 TVFA may be the key factor that leads to long-term effects on rumen environment in calves fed MR.

(1) The nutrient value and digestibility of MR were lower than that of W and WM. This may result in a decreased growth rate in preweaning calves fed MR. However, calves tend to consume more starter feed to compensate nutrient deficiency. Carbohydrates in starter feed can be rapidly fermented into VFA, which may promote epithelium proliferation and contribute to a diverse microbiota establishing in rumen.

(2) The higher TVFA in rumen can also be attributed to a poor absorption of rumen epithelium. Liquid feed may enter the rumen due to the insufficient closure of oesophageal groove or abomasum reflux. The presence of soy protein may negatively affect rumen epithelium and leads to a poor absorption efficiency of VFA. The reduced absorptive ability of GI tract in calves fed soy protein might be associated with morphological abnormalities, such as the villous atrophy with abnormal mucosae (Seegraber and morrill, 1986).

1.3 The antimicrobial activity of SAG may induce differences in ruminal bacterial community; The increased Desulfovibrio might be associated with anti-inflammation property of SAG; Protein utilization can be enhanced by dietary SAG.

(1) The wide spectrum of antimicrobial activities of sanguinarine may decrease bacterial richness and lead to a reduction in relative abundance of genus *Prevotella 1*, *Butyrivibrio 2*, *Prevotellaceae UCG-003*, and *Prevotellaceae UCG-004* in the rumen of calves fed SAG.

(2) *Desulfovibrio* accounts for the majority of dissimilatory sulfate-reducing activity in the rumen. It converts sulfate to hydrogen sulfide (H_2S), which plays an important role in mucosal defense in digestive system (Wang et al., 2002; Kimura et al., 2005). SAG has been demonstrated to possess prominent anti-inflammatory effects (Niu et al., 2012). We observed that feeding SAG increased the population of *Desulfovibrio* in the rumen of calves, which was in accordance with a recent research in fish (Zhang et al., 2019). Thus, we speculated that anti-inflammatory activity of SAG might be associated with the increased population of *Desulfovibrio* and its metabolite H_2S in GI tract. However, the anti-inflammatory activity was not measured in this research. Further researches are needed to validate this hypothesis.

(3) Feeding SAG to calves decreased the concentration of NH_3-N

in rumen. This can be explained as below: Firstly, SAG could increase flow of non-ammonia N to small intestine, which decreased feed protein degradation and increased microbial protein synthesis in cattle and steers (Plascencia and Zinn, 2014; Aguilar-Hernández, et al., 2016). Secondly, SAG could decrease amino acid degradation from decarboxylation (Drsata et al., 1996). Thirdly, SAG may exert antimicrobial effects on high ammonia-producing bacteria.

1.4 RES increased Desulfovibrio whereas decreased methanogenic bacteria. It might be a promising additive to reduce methane production.

(1) Feeding RES to calves affected rumen fermentation parameters and bacterial community, which was in accordance with previous studies (Becker and Van Wikselaar, 2011; Ma et al., 2015).

(2) Feeding RES to calves increased the population on *Desulfovibrio*, which was reported to compete with methanogenic bacteria for substrates such as H_2 and acetate (Bryant et al., 1977; Oremland et al., 1982). Previous studies indicated that dietary RES decreased the population of methanogenic bacteria methane output (Ma et al., 2015). Therefore, we further quantified the population of methanogenic archaea, and found it was decreased in calves fed RES. It suggested that RES might be a promising additive to reduce methane production.

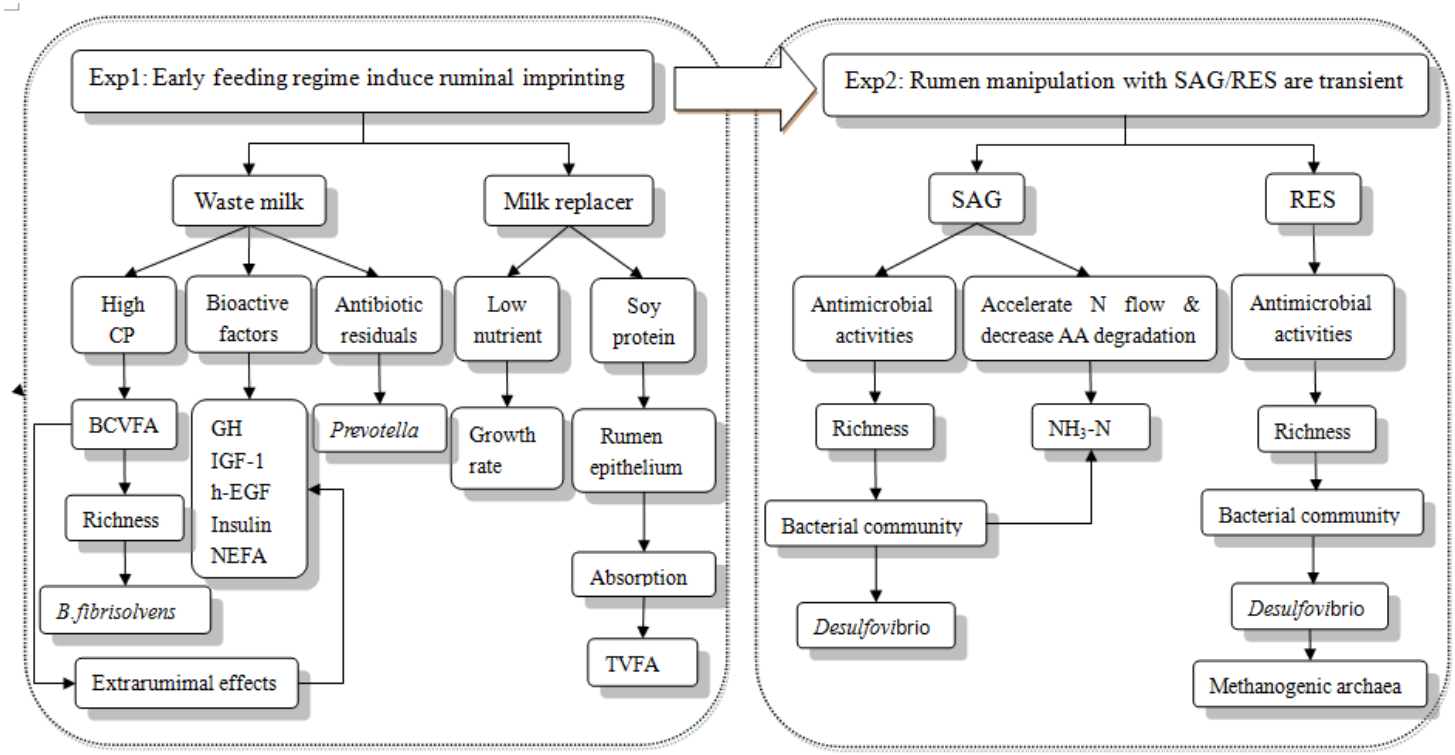

Figure 19. The overview of general discussion

2. Conclusion

Numerous attempts to manipulate fully matured rumen environment proved to be invalid. The feasible “window” for rumen microbial programming may exist in the early life of ruminants when the microbiota is establishing. Nutritional manipulation during these time may produce a more efficient and sustainable effect. Our results demonstrated that liquid feeding regime and nutrition impacted rumen development not only by dry matter intake, but also the type of liquid feed. The type of liquid feed may affect the life-time performance of cattle by influencing rumen development and bacterial composition.

In this research, we observed that calves fed WM had a decreased rumen weight and a greater molar proportion of ruminal isovalerate, which altered the bacterial community in the rumen and induced an extraruminal effect in the blood. WM seems to be more beneficial for the growth of preweaning calves. However, the effect of WM on dairy calves is largely dependent on its composition. The information of nutrient profiles, antibiotic residuals, and antibodies in WM should be investigated before it can be fed to calves. It can be inferred from our results that growth hormones and bioactive factors, such as GH and IGF-1, could be effective additives to promote the rumen epithelial cell proliferation. And isoacids, such as isovalerate and isobutyrate, could be used to improve the diversity of rumen bacteria, and to increase the population of cellulolytic bacteria, which may facilitate calves to digest forage more efficiently.

The nutrient level and ingredient composition of MR are the main factors that determine the growth rate of calves. Generally speaking, the fat content of MR could not reach that of whole milk, thus calves tend to consume more starter feed to compensate the energy shortage. Carbohydrates in starter feed can be rapidly fermented into VFA, which may be a key factor that contributes to diverse microbiota establishing in the rumen. The use of plant proteins in MR may reduce the VFA absorption of rumen epithelium and thus induce microbial programming effects in rumen. Therefore, a long-term effect of VFA supplementation on rumen development in calves should be determined in the future. And effects of all-milk-protein MR and soy-protein MR on the absorption of rumen epithelium also deserve further researches.

Dietary supplementation of sanguinarine or resveratrol to calves affected the rumen fermentation environment for a short time. The population of Desulfovibrio was increased by sanguinarine and resveratrol, whereas the population of methanogenic archaea was decreased by resveratrol. However, no persistence of any effects was observed on rumen environment in calves feeding sanguinarine or resveratrol, as both rumen fermentation parameters and the bacterial community were restored to their original state prior to 6 months of age. Sanguinarine and resverotrol have extensive biological properties,

which include anti-inflammatory and antioxidant activities. These biological characteristics may be closely related to bacterial community in the GI tract of calves. In this research, we observed that feeding resveratrol to calves decreased the population of methanogenic archaea in rumen. Resveratrol might be a promising feed additive to reduce methane emissions. The effect of dietary resveratrol on methane production also deserves further researches. Finally, the population of protozoan and communities of archaea in rumen should be investigated along with bacteria for a better explanation.

3. Perspectives

Rumen is the most important digestive organ that plays an important role in growth, production performance and health of ruminants. Promoting rumen development has always been a key target of calf nutrition. More and more researches reveal that early feeding regime and nutrition have effects on rumen development and the establishment of rumen microbiota. The type of liquid feed differently affects rumen bacterial composition and fermentation profiles. The effects may persist for a long time, and consequently, impact the life-time productive performance and health of the adult ruminants. As a benefit, this may suggest a possibility of rumen microbial programming.

Nutritional interventions can represent a vital determinant of growth and health of adults by influencing the establishment of rumen microbiota. The most sensitive window for rumen manipulation may exist in postnatal and weaning period. Jami et al. (2013) indicated that cellulolytic bacteria and other bacterial species important to rumen function can be detected as early as 1 day after birth. The presence of cellulolytic and methanogenic bacteria was observed in lambs at 3-4 days of age, and the population of these bacteria reached a level similar to that observed in mature sheep within 7 days of age (Fonty et al., 1987). Thus, the potential critical windows for microbial programming by dietary intervention might be more effective during the 1st week of age. However, the efficancy of early nutritional interventions are subjected to the type of intervention and its duration. With increasing age, the anatomical volume of the rumen increases rapidly and the richness and diversity of rumen bacteria are enhanced dramatically, which might eliminate the impact of early nutritional intervention. Additionally, the introduction of pellet feed, roughage and changes in diet composition during different stages may affect rumen microbiota and fermentation environment, which may be redirectional processes for rumen microbiota. This may also impair the long-last effects of early nutritional intervention.

The crosstalk between host and microbes in the rumen plays an important role in shaping host performance. Microbes residing in the rumen digest and ferments feeds into nutrients that are subsequently utilized by the host. The metabolites in rumen participate in a variety of

physiological processes of the host. Saleem et al. (2013) found 246 metabolites in ruminal fluids, including phospholipids, inorganic ions and gases, amino acids, dicarboxylic acids, VFA and carbohydrates. VFA are the major bacterial metabolites in rumen, which meet 70-80% of the energy requirement for the rumen epithelia, and 50-70% the energy requirement for the animals (Bergman, 1990), thereby acting as important drivers in the ruminal microbiome-host crosstalk. The microbe-G-protein-coupled receptor (GPR) and microbe-histone deacetylase (HDAC) axes may be one of the major pathways mediating the effects of VFA on epithelium growth and metabolism (Shen et al., 2017). Exogenous butyrate promotes the expression of genes associated with cell growth, signal transduction, and immune responses in bovine rumen epithelium cell cultures (Li et al., 2016). An integrated analysis in ruminal microbiota and host transcriptomic profiles of lambs revealed that the increased concentrations of acetate and butyrate in rumen were significantly correlated with the expression of growth-related genes (Lin et al., 2019). Additionally, the metabolites and microbial colonization may affect gene expression of miRNAs in rumen epithelium, and thus regulate the development of the host immune system. High expression of miR-143 throughout the GI tract in calves is proposed to be associated with the rapid development and growth of the GI tract during early life (Liang et al., 2014).

Host genetics may contribute to individual variations in rumen microbiota and fermentation dynamics. Rumen bacterial communities presented low similarity across 16 calves raised under similar housing conditions and diets (Jami and Mizrahi, 2012). The rumen microbial re-establishment patterns were unique for individual animals after rumen being emptied and receiving content from donor (Zhou et al., 2018). Rumen function and host productivity can be predicted from a small number of core microbes (Sasson et al., 2017). A core rumen microbiome, phylogenetically linked and with a preserved hierarchical structure, was identified in a 1000-cow study (Wallace et al., 2019). Therefore, the core heritable rumen bacteria may be served as the primary target for rumen bacteria manipulation in the future.

Rumen development is driven by both host and microbes. However, the current understanding of the establishment of rumen microbiota and their interactions with the host remains very limited. Previous researches of calves were mainly focus on growth, blood metabolites and rumen fermentation. Amplicon sequencing and shotgun metagenomics are the most commonly used technologies for rumen microbiota investigation. However, the use of relative abundance of microbes may also limit our understanding of rumen microbial ecology. Shi et al. (2014) reported strong correlations between the expression levels of the hydro-genotrophic methanogenesis pathways in rumen methanogens and methane yields, in the absence of changes in methanogen community structure. The relationship between relative

transcript abundance and protein is also not straightforward (Hart et al., 2018). The integration of omics technologies including metagenome, metatranscriptome, metaproteome and metabolomics enable us to learn more about the structure and function of the dynamic and complex microbial community.

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