Effects of Early Weaning on the Rumen Bacterial Diversity in Dairy Lambs

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Research Article

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Abstract

Background

The early weaning (EW) technology is an efficient method to improve the economic value of dairy ewes and satisfy the nutritional requirement of lambs which due to multiple fetuses or insufficient milk production of ewes. However, the affection of EW for rumen microflora is needed to evaluation because of the rumen microflora at early life would affect the health of host at whole life. In this study, the rumen microflora of dairy sheep with different EW time (D7, D15 and D25) were analyzed through 16s rDNA sequencing at D90.

Results

The results showed that the application of EW did not affect the development of rumen. The species richness of the EW25 group was higher than EW7 and EW15 groups, while there was no significant difference in the abundance of microbial species between the CON and EW groups. At the phylum level, Firmicutes, Bacteroidetes, Fibrobacteres, Spirochaetes, Proteobacteria and Actinobacteria were the most dominant phyla in all the samples. At tenricutes level, the relative abundance of EW7 group was significantly higher than other three groups (p<0.05). The spearman correlation coefficient analysis showed that pH was positively correlated with Rikenellaceae_RC9, Succinivibrionaceae, F082_unclassified, etc. (p<0.05), and negatively correlated with Ruminococcaceae_UCG-014, Succinivibrionaceae, Selenomonas, etc. (p<0.05). The content of MCP was positively correlated with CAG-352 and Ruminococcus_2 (p<0.05). The thickness of rumen muscle layer was positively correlated with Sharpea (p<0.05).

Conclusions

In summary, the EW of dairy lambs does not affect the development of rumen at early life. The rumen bacterial diversity showed some differences between different EW days of age but no obvious effect, which could be a reference for dairy lamb’s EW time selection.

Background

The rumen is a unique digestive organ of ruminants, plays an important role in degradation of fibrous matters. The degradation of fibrous matters relies on microorganisms of rumen. Rumen microorganisms can supply about 70% of energy for the requirement of hosts through fermentation [1]. The stability of the rumen microbiota is essential for the health of hosts and the performance of production. The change of rumen microflora related to animal productivity closely [2]. The breeds and age of animals are the direct affective factors of rumen microflora [3, 4]. And the feedstuffs is one of the most important affective factors of rumen microflora [5]. In addition, the supplement of additives can improve rumen microbial community structure and improve the production efficiency [6].
After birth, the lambs face a weaning transition and dietary changes from milk or milk replacer (MR) to a solid diet. During this period, the gastrointestinal tissues would change from metabolizing glucose to fatty acids, especially in rumen. Traditionally, the lambs were suckled with the mother until 2 months old. However, it would prolong the breeding cycle and impact the body recovery, and then reduce reproductive efficiency, especially for dairy breeds [7]. On the other hand, it would affect the healthy growth and development of lambs because of the breast nursing can not satisfy the nutritional requirement due to multiple fetuses or insufficient milk production of ewes [8]. The early weaning (EW) technology is an efficient way to solve these problems.

However, the affection of EW for rumen microflora is needed to evaluation. In the present study, we used DNA amplicon sequencing of the 16S rRNA gene in the rumen digesta of dairy lambs raised on MR & starter of breast milk & starter and aimed to investigate the effects of MR on the rumen microbiota.

**Results**

**Rumen phenotypic analysis**

The rumen phenotypic results of all the groups were shown in Table 1. There were no significant difference (p > 0.05) in the pH, MCP, papillae length and muscle layers thickness between different groups. The papillae width of lambs in EW25 group was significant lower than other groups (p< 0.05). The result indicated that there was little affection of EW on rumen development of dairy lambs.

**Sequences Analyses**

After sequencing and filtering low-quality and long-section sequences, 785430 counts were obtained from 14 samples. A total of 988806 valid sequences were obtained after quality control. The length of most sequences is between 400-500bp (99.93% of the total).

A total of 9481 OTUs were obtained using QIIME2. Among them, 9482, 8428, 9105 and 8252 OTUs were obtained from EW7, EW15, EW25 and CON group, respectively. There were 140 OTUs were shared by all four groups (Figure 1A). 2038, 1091, 3115 and 2015 OTUs were unique at EW7, EW15, EW25 and CON group, respectively (Figure 1A). The rarefaction curves of all samples tended to be flat, which indicated that the sequencing data volume is reasonable (Figure 1B).

**Alpha diversity analysis**

As shown in Figure 2, the Chao1, observed species, Shannon of group EW25 were significantly higher than group EW7 and EW15 (p<0.05). There was no significant difference of Simpson among all group (p>0.05). It showed that the species richness of the EW25 group was higher than EW7 and EW15 groups, while there was no significant difference in the abundance of microbial species between the CON and EW groups. The result indicated that there was no distinct affection of EW on rumen microbial species abundance of dairy lambs.
**Beta diversity analysis**

ANOSIM similarity analysis showed that the test was reliable (p=0.00041) and the difference between groups was higher than the difference within group (R=0.476608) (Figure 3A).

The Principal Coordinates Analysis (PCoA) analysis based on the unweighted unifract distance matrix of multiple communities was used to judge the similarity of species composition among the samples. The results showed that there was significant difference between EW7 and EW25 groups (p<0.05), the rest showed no significant difference (p>0.05) (Figure 3B).

The cluster analysis showed that the species composition and richness of different groups were similarly, just there was a difference between CON and EW15 groups (Figure 3C).

**Bacteria composition analysis**

A total of 22 phyla were detected in this study. Among them, there were 9 core flora with relative abundance greater than 0.1%. The distribution of the dominant microbial flora at the phylum level was shown in Figure 4A. The top six common dominant bacterial groups were *Firmicutes, Bacteroidetes, Fibrobacteres, Spirochaetes, Proteobacteria* and *Actinobacteria*, of which the relative abundances were all greater than 1%. The 30 most abundant microbial species at the genus level were shown in Figure 4B. The *Prevotella* and *Fibrobacter* were dominant genera in four groups.

At tenericutes level, the relative abundance of EW7 group was significantly higher than other three groups (p<0.05) (Figure 5A). The relative abundance of *Planctomycetes* of EW25 and EW15 groups were higher than other two groups (p<0.05) (Figure 5B). The relative abundance of *Fibrobacteres* of EW7 group was lower than CON group (P<0.05) (Figure 5C).

At genus level, the relative abundance of *Acidaminococcus* of EW7 group was significantly higher than EW25 group (p<0.05) (Figure 6A). The relative abundance of *Fibrobacter* of EW7 group was significantly lower than CON group (p<0.05) (Figure 6B).

**LEfSe analysis of intergroup samples**

Based on the OTUs level, the specific microorganisms between the 4 groups were summarized through LEfSe analysis and the filter value of LDA Score is 4. Most of bacteria were non-specific and a few statistically and biologically biomarkers belonged to specific bacterial clades (Figure7A). The LDA histogram showed that only 7 specific bacteria genera were significantly enriched in the EW7 group includes *Succinivibrionaceae* and *Aeromonadales*, 5 specific bacteria genera were significantly enriched in CON group includes *Fibrobacteraceae* and *Fibrobacterales* (Figure7B).

**Relationship between rumen phenotypes and bacterial community**

The spearman correlation coefficient was used to study the correlation between rumen phenotypes and bacterial species at the genus level (Figure8). Results showed that pH was positively correlated with
Rikenellaceae_RC9, Succiniclasticum, F082_unclassified, etc. (p<0.05), and negatively correlated with Ruminococcaceae_UCG-014, Succiniclasticum, Selenomonas, etc. (p<0.05). The content of MCP was positively correlated with CAG-352 and Ruminococcus_2 (p<0.05). The width of rumen papilla was negatively correlated with non-classifiable bacteria (p<0.05). The thickness of rumen muscle layer was positively correlated with Sharpea (p<0.05).

**Discussion**

The microbiome of the rumen at infancy has attracted more and more attention because of its potential relationship with long-term impact on an animal's development and performance [9, 10]. Understanding the rumen microbiota is important to elucidate its potential role in rumen development. It has been suggested that the development of rumen microbiota begins with the intake of solid feed and precedes the development of rumen [11]. In this study, we found that the development of rumen was not affected by MR supplement. Perhaps the supplement of leymus chinensis and strater from D7 has promoted the development of rumen at all groups. The papillae width of lambs in EW25 group was significant lower than other groups. This is similar to previous report, in which the dietary concentrate level increased, and the rumen papillae width decreased [12].

The top six common dominant bacterial groups were **Firmicutes**, **Bacteroidetes**, **Fibrobacteres** and **Proteobacteria** in lamb's rumen. The **Firmicutes**, **Bacteroidetes** and **Proteobacteria** are dominant bacteria at any ages [10, 11, 13]. The **Firmicutes** are thought to be the main flora that promotes cellulose breakdown by host gastrointestinal microbes [14-16]. The **Bacteroides** are considered to be the most important microflora that promotes carbohydrate utilization in the host [17-19]. The **Proteobacteria** includes many bacteria that fix nitrogen, which could be nitrogen a sourse of nitrogen of hosts [20]. The **Fibrobacteres** and **Sucinivibrio** accelerate rumen fermentation by destroy the plant cytoderm and degrade the fiber structure, and then facilitate the rumen microbial colonization [21]. These bacteria could utilize starch, other non-cellulose polysaccharides and monosaccharides as energy sources to produce succinic acid as the main fermentation end product. Their role in ruminant physiology is particularly significant because of succinic acid decarboxylates rapidly in the rumen and the resulting propionic acid plays a key role in the maintenance of glucose homeostasis in hosts through gluconeogenesis [22]. This study found that a significant difference of species to decompose fiber type. Compared with CON group, the **Fibrobacteres** of EW7 group were significantly lower. But its soft wall fungus phylum present relatively high abundance, which suggests that the relative weakness of hay utilization of EW7 lambs, while relative better fodder utilization.

**Conclusion**

In summary, the EW of dairy lambs does not affect the development of rumen at early life. The rumen bacterial diversity showed some differences between different EW days of age but no obvious effect, which could be a reference for dairy lamb's EW time selection.
Materials and Methods

Ethics statement

The study protocol was reviewed and approved by the Animal Welfare Ethics Committee of Inner Mongolia University (Approval No. 2018003). And the animal procedures used in this study strictly abide by the Administrative Measures of Gansu Province on Experimental Animals.

Animal study and sample collection

Twenty-six male F1 lambs (5.07±0.22kg of birth weight, similar birth age) which crossbred with Small-Tailed Han (STH) sheep as female parent and DairyMeade (DM) sheep as male parent were sourced. Six lambs of each EW group and eight of control (CON) group. All of lambs were separated from their mothers at D7 after birth. The temperature of breeding house was -4 ~ 30℃. All lambs were supplied with the leymus chinensis and strater from D7. The lambs of CON group were artificially-reared with breast milk to D60. The lambs of EW groups were artificially-reared with MR from D7 (EW7), D15 (EW15) and D25 (EW25) to D60. Four days were needed to use MR completely, 25% percent of volume of MR was added every day. The MR was reconstituted at 200 g/L of water and offered at a temperature of ~40 °C. Four times per day of feeding before D30 and followed three times per day till D60. The nutritious components of MR and starter were listed at Table 1. After D60, All lambs were weaned off liquid supplements and fed with solid fodder totally. The warm water (10~20°C) was supplied anytime.

At D90, three to five lambs of each group were slaughtered and the rumen were collected. The pH of rumen was measured immediately. The rumen content were mixed well and collected into freezer-storage tubes immediately and stored at -80°C for DNA extraction and further analysis. The rumen abdominal sac tissue were rinsed and fixed in 4% paraformaldehyde for histomorphological analysis (ruminal papillae length and width and the tunica muscularis thickness).

Detection of rumen MCP and rumen tissue morphology

The microbial crude protein (MCP) was detected by Multifunctional fluorescence detector (Thermo scientific). The length, width and muscular thickness of the rumen papilla were measured using a Digital microscope (Nikon).

DNA extraction, high-throughput sequencing and data procession

The bacterial genomic DNA analysis was performed by LC-Bio Co., Ltd (Hangzhou, China).

Total genomic DNA from the samples was extracted using the E.Z.N.A.®Stool DNA Kit (Omega, Guangzhou, China) following the producer’s instructions. DNA quality was determined by electrophoresis on agarose gel 0.8% (w/v) and the quantity was determined using a NanodropTM spectrophotometer (NanoDrop Technologies, Thermo Scientific, USA).
Specific PCR primers of 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') were synthesized and Phusion R High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, United States) was used to amplify the V3/V4 regions as described by Caporaso et al [23]. The amplicon libraries for all samples were pooled at an equimolar ratio and sequenced on an Illumina HiSeq platform by LC-Bio Co., Ltd.

The QIIME software (Qiime1.9.1) was used for 16S rRNA original data quality preliminary screening and analysis. Interrogative and short sequences (<200 bp) were discarded using the QIIME software. The obtained sequences were clustered and operational taxonomic unit (OTU) partitioned at ≥97% sequence similarity by using the clustering program VSEARCH (1.9.6.). A representative sequence of each OTU was classified at a confidence threshold of 0.8 according to the Ribosomal Database Program (RDP) classifier. Additionally, the obtained sequences with 97% similarity were merged to the same OTUs. Before calculating alpha and beta diversity statistics, the sequencing depth of each sample was evaluated using sparse curves. Continuous analysis of alpha diversity and beta diversity were performed based on the output normalized date. Four metrics including Chao1, ACE, Simpson, and Shannon were used to analyze alpha diversity. GraphPad Prism 6.0 and R (v3.0.3) software were used for statistical analysis[24]. The criterion of significance was performed at p < 0.05 and the values were presented as means ± SEM.

**Abbreviations**

CON: Control; DM: DairyMeade; EW: Early Weaning; MCP: Microbial Crude Protein; MR: Milk Replacer; OTU: Operational Taxonomic Unit; PCoA: Principal Coordinates Analysis; RDP: Ribosomal Database Program; STH: Small-Tailed Han.

**Declarations**

- Ethical Approval and Consent to participate

The sheep trial and all procedures for sampling and measurement taking was approved by the Animal Welfare Ethics Committee of Inner Mongolia University (Approval No. 2018003).

- Consent for publication

Not applicable.

- Availability of supporting data

All data generated or analysed during this study are included in this published article.

- Competing interests

The authors declare that they have no competing interests.
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- Authors' contributions

Li Zhang and Guangpeng Li designed the study. Ying Ma, Zhong Zheng, Liguo Zhang, Yingjie Dou, Xiaoran Zhang, Urhan Bai and Guanghua Su ran the trial and sample collection. Ying Ma and Xiaohu Su performed the DNA extractions. Xiaohu Su and Ying Ma analysed the data and interpreted the results. Xiaohu Su wrote the manuscript. The authors have all read and approved the final version of the manuscript.

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Tables

Table 1. Nutritional levels of milk replacer and starter (%)

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| Item           | Milk replacer (MR) | Starter |
|---------------|--------------------|---------|
| Crude Protein | 23                 | 18      |
| Crude Fiber   | 3                  | 9       |
| Crude Ash     | 10                 | 12      |
| Ca            | 0.6~1.5            | 0.1~1.8 |
| Total P       | 0.5~1.2            | 0.3     |
| NaCl          | 0.1~1.2            | 0.6~1.2 |
| Lys           | 2.2                | 0.4     |

Table 2. Rumen phenotype of lambs.

| Items                        | EW7                  | EW15                 | EW25                  | CON                  |
|------------------------------|----------------------|----------------------|-----------------------|----------------------|
| pH                           | 6.55±0.22            | 6.81±0.14            | 6.89±0.34             | 6.55±0.22            |
| MCP (mg/mL)                  | 2.70±0.11            | 2.29±0.21            | 2.47±0.03             | 2.83±0.16            |
| Papillae length (µm)         | 1486.55±111.99       | 1398.35±64.13        | 1265.14±177.22        | 1627.52±82.23        |
| Papillae width (µm)          | 232.59±12.72<sup>a</sup> | 204.63±8.37<sup>a</sup> | 180.84±6.33<sup>b</sup> | 226.24±12.29<sup>a</sup> |
| Muscle layers thickness (µm) | 1339.28±43.67        | 1159.91±68.93        | 1221.81±51.5          | 1100.17±105.71       |

Adjacent letters on the shoulders of peers indicate significant difference (p<0.05).

**Figures**
Figure 1

1 Number of operational taxonomic units (OTUs) in each group. (A) Venn diagram of shared OTUs. (B) Rarefaction curves of OTUs.

Figure 2

Effects of early weaning on rumen microorganism α diversity of lambs. (A) Chao 1. (B) Observed species. (C) Shannon. (D) Simpson.
Figure 3

Unweighted Unifrac distance based on the Principal coordinate analysis (PCoA). (A) Anosim analysis. (B) Unweighted Unifrac distance based on the Principal coordinate analysis (PCoA). (C) UPGMA (unweighted pair group method with arithmetic mean) clustering graph.

Figure 4

Effect of early weaning on the rumen bacteria abundance. (A) Phylum level. (B) Genus level. Each bar represents the average relative abundance of each bacterial taxon within a group.
Figure 5

Dominant strains with significant differences in phylum level. (A) Tenericutes. (B) Planctomycetes. (C) Fibrobacteres.

Figure 6

Dominant strains with significant differences in genus level (A) Acidaminococcus. (B) Fibrobacter.
Figure 7

Cladogram and linear discriminant analysis. (A) Cladogram. (B) Linear discriminant analysis (LDA). Bacterial taxa significantly differentiated between the C and S groups identified by LDA coupled with effect size (LEfSe) using the default parameters.

Figure 8

Spearman's correlation between the rumen bacterial communities (genus level) and phenotypic variables. Red represents a positive correlation; blue represents a negative correlation and yellow represents a weak correlation. *p < 0.05, **p< 0.01.