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SCIENTIFIC PRESS PTE.LTD.

Molecular & Cellular Biomechanics 2024, 21(2), 439.
https://doi.org/10.62617/mcb439

Article

Effects of weaning stress and Bacillus licheniformis intervention on rumen and intestinal microflora of Hu lambs

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CITATION

Li S, Hashim Z. Effects of weaning stress and Bacillus licheniformis intervention on rumen and intestinal microflora of Hu lambs. Molecular & Cellular Biomechanics. 2024; 21(2): 439.
https://doi.org/10.62617/mcb439

ARTICLE INFO

Received: 29 September 2024
Accepted: 24 October 2024
Available online: 7 November 2024

Abstract: The aim of this experiment was to investigate the effect of early weaning on the diversity of rumen and intestinal microbiota in Hu lambs, and the role of adding Bacillus licheniformis to the ration in regulating weaning stress in lambs. Ninety newborn Hu lambs with natural delivery and birth weight close to (3.82 ± 0.46 kg) were selected for the experiment, and were randomly divided into three treatment groups: normal weaning group (CON group, 49 d weaning), early weaning group (EW group, 21 d weaning), and B. licheniformis group (BL group, fed with 60 mg/kg BW B. licheniformis, viable count ≥ 2×10^9 cfu/g, weaned at 21 d), were slaughtered at 26, 35, and 63 d, rumen contents, rumen fluid samples, and jejunal segments were collected for subsequent experiments. The results showed that weaning stress reduced the abundance and diversity of flora in the rumen and jejunal contents and mucosa of lambs in the short term, but allowed the flora to enter a steady state earlier without affecting the final flora abundance and diversity, early feeding of B. licheniformis helped to restore the abundance of some genera in the rumen and jejunum of lambs.

Keywords: early weaning; lambs; Bacillus licheniformis; microorganisms

1. Introduction

In recent years, Hu sheep have been introduced in large quantities by farmers because of their high fertility. However, under the background of "banning antibiotics in feed", how to improve the environmental adaptability and disease resistance of introduced sheep through antibiotic substitutes has become an important issue in animal husbandry. Probiotics, as green feed additives, are widely used in livestock and poultry production due to their safety and efficiency, no residue, and no drug resistance, but the effect of their addition in ruminant diets needs a lot of research. Therefore, this experiment aims to study the effects of weaning stress and the addition of B. licheniformis on the rumen microbiota and gut microbiota of young ruminants will provide basic data for the application of B. licheniformis-like probiotic enzymes in the healthy culture of ruminants.

2. Literature review

Early weaning can have serious effects on the growth and health of young ruminants, and nutritional regulation by taking some necessary measures, such as feeding management, dietary supply and feed additives, can alleviate weaning stress in lambs and reduce the potential harm caused by stress to young ruminants [1]. Probiotics are live microorganisms that have been rigorously selected for their health

benefits to the host and are also known as direct-fed microorganisms [2]. Studies have shown that probiotics contribute to normal digestion, support the immune system and promote overall health, help maintain a normal balance in the gut, and are effective in preventing and treating disease primarily by modulating mucosal immune activity and epithelial barrier function as a bio-antagonist [3]. Commonly used probiotic species include Lactobacillus species, yeast species, Bacillus species, and probiotic complexes. For ruminants, probiotics should fulfill the following conditions: able to survive low pH conditions in the rumen and tolerate bile acids, beneficial to the animal organism, has the ability to adhere to intestinal epithelial cells and colonize the intestines permanently or periodically, able to be easily stored and processed under normal conditions [4]. Studies on piglets have shown that probiotics function mainly by inhibiting the growth and adhesion of pathogenic bacteria in the gut, stimulating the development of the immune system in piglets or modifying the intrinsic intestinal flora [5]. B. licheniformis as a feed additive has been reported in many studies in the production of poultry and aquaculture, etc., and has been widely used mainly as a growth promoter and competitive exclusion agent. The suitability of Bacillus sp. for use as a feed additive is mainly due to its higher resistance to harsh environments and its ability to produce a wide range of enzymes including proteases, amylases and lipases [6]. By producing anti-microbial actives and reacting with oxygen, it delays the multiplication of pathogens, enhances body immunity and improves intestinal health [7,8].

Early nutritional intervention has been suggested as an effective means to manipulate the rumen microbiome. Studies have shown that the early stages of young animals may be a window of opportunity for regulating rumen microbiota colonization, and that nutritional interventions at early stages of life may have an impact on rumen flora composition [9]. Feeding of B. licheniformis promoted an increase in the proportion of Bifidobacterium spp. Bifidobacterium spp. are lactic acid fermenting bacteria that inhibit pathogenic microbial infections by enhancing humoral immunity, and are the predominant bacteria in the intestinal flora of breastfed infants, and are capable of preventing or mitigating contagious diarrhea, which suggests that B. licheniformis may also have the same ability to improve the composition of the jejunum flora in lambs and increase the colonization of beneficial bacteria [10]. Currently, it has been shown that B. licheniformis can improve the performance and health of piglets and poultry, but there are fewer reports on the application of B. licheniformis in young ruminants [11,12].

3. Experimental materials and methods

3.1. Test animals

In this experiment, Hu lambs were selected as test materials and were reared in Ningxia Nongken Helanshan Cattle and Sheep Industry Group. From the newborn lambs, 90 Hu lambs were selected which were born naturally, the difference of birth date was not more than 3 d, the birth weight was close to (3.82 ± 0.46) kg, the body condition was good, and the health was free of disease, and the difference of weight of each group after grouping was not significant ($P > 0.05$).

3.2. Experimental design

The lambs were randomly divided into 3 groups of 3 replicates of 10 lambs each, grouped in such a way as to ensure that the weights of the lambs in each group were similar, as well as that the number of individuals in each group was comparable between male and female lambs. The three groups were control group (CON group, 49 d weaning), early weaning group (EW group, 21 d weaning), and B. licheniformis group (BL group, fed B. licheniformis, 21 d weaning). Each group had 3 replicates of 10 lambs (8 males and 2 females). The total experimental period was 63 d.

From day 4 onwards, additive feeding was carried out every morning at 8:00 a.m. All three groups of lambs were fed with natural lactation plus supplemental milk replacer. Lambs in the EW and BL groups were weaned at 21 d. Control lambs were nursed with their mothers until 49 d of weaning B. licheniformis by Xin Dayang Biotechnology Co., Ltd, strain conservation code: CGMCC NO.7.54 lichen, viable bacterial number $\geq 2 \times 10^9$ cfu/g, the carrier is glucose, the recommended dosage of 2 g/kg, that is, 4×10^9 cfu/kg of feed. The specific additive doses for the trial were: 300 mg/d per lamb, i.e., 0.6×10^9 cfu/d of live bacteria, during 4–35 d, and 600 mg/d, i.e., 1.2×10^9 cfu/d of live bacteria, during 36–63 d.

3.3. Ration and feeding management

The sheep house was a semi-open pen with sufficient light area and good ventilation. All test lambs were ear-numbered after 3 d of life and immunized according to the farm's vaccination program. After birth, the lambs were kept in the same pen with the ewes and nursed with the ewes, while the lambs in the EW and BL groups were separated from the ewes at 21 days of age (lambs stayed in the same place and ewes were removed) and weaned in one go, In the CON group, the lambs continued to nurse with their mothers at 21 days of age, and at 49 days of age, the lambs were separated from their mothers (the lambs were left in the same place, and the mothers were moved away) and weaned at one time. During the test period, the lambs were fed and watered ad libitum, and no antibiotics were used in the diet or in the feeding process.

3.4. Sample collection

Samples were taken at the time of slaughter at 26 d, 35 d, and 63 d. Lambs were collected for rumen contents and 2-cm-long intestinal segments from the mid-jejunum. The rumen contents were removed and mixed homogeneously, and the pH was determined. 3 tubes of rumen contents were taken in liquid nitrogen using 2 mL cryopreservation tubes and stored in a refrigerator at −80 ℃ for determination of the rumen microbiota. After the rumen contents were filtered through four layers of gauze for rumen residue, 10 mL of rumen fluid was taken and stored at −20 ℃ for determination of VFA and NH3-N content. About 2 cm long jejunal segments were placed on a flat ice surface, cut longitudinally, washed with PBS buffer to remove the chyme, rinsed with PBS for 3 times, and the mucosa was gently scraped off with a clean slide and loaded into a freezing tube, the contents of the middle part of the jejunum of the lambs were collected in 2 mL freezing tubes, quickly placed in liquid nitrogen and stored at −80 ℃ for determination of the microbiota.

3.5. Indicators and methods for ruminal fermentation and ruminal flora

3.5.1. Determination of rumen fermentation parameters

Ruminal pH determination was performed using a portable pH meter. The VFA concentration in the samples was determined according to the method provided by Zhang et al. [13], which consisted of adding 1 mL of rumen filtrate to 0.2 mL of metaphosphoric acid solution (250 g/L) containing 2 g/L butyric acid-2-ethyl ester, mixing overnight, and detecting the concentration of VFA by gas chromatography (SP-3420, Beijing Analytical Instrument Factory, Beijing, China). Detection NH3-N concentration was determined by the phenol hypochlorite colorimetric method following the method in the literature [14].

Samples were subjected to DNA extraction using the Omega Stool DNA kit. After DNA extraction, DNA quality and concentration were determined by 1% agarose gel electrophoresis and spectrophotometry, and quality-checked samples were stored at −20 ℃ for subsequent experiments.

3.5.2. High-throughput sequencing analysis to determine microbiota

(1) PCR amplification and product purification: The sample DNA was diluted to 1 ng/μL with sterile water, and the bacterial 16S rDNA gene was amplified in the V3-V4 sequencing region using synthesized specific primers using the barcode as a template. the gel was recovered after the PCR amplification reaction using a gel recovery kit. Library construction was performed using the TruSeq® DNA PCR-Free DNA Library Kit and constructed libraries were tested by Qubit and then sequenced using the HiSeq 2500 PE250 [15]. After binning the raw data from sequencing, the results are stored in Fastq format, and the offline data (Raw PE) from barcodes and primers are spliced to obtain raw tags, which are further processed into high-quality clean tags for sequences.

(2) Species annotation: Operational taxonomic units (OTUs) were generated by cluster analysis of all valid tags for all samples with 97% sequence identity level using U parse software [16]. The highest frequency sequences in the OTUs were selected as representative sequences of the OTUs, and the representative sequences of the OTUs were compared with the Green Genes bacterial database using a Bayesian algorithm (RDP classification version 2.2) to count the composition of the bacterial groups at each taxonomic level in each sample and to calculate the relative abundance of bacteria for species annotation.

(3) Complexity analysis of the sample flora: Alpha diversity indices including Observed-species, Shannon index, Chao1 index and PD whole tree index were statistically analyzed using Qiime version v.1.8.0 software [17].

3.6. Data processing

The data were statistically analyzed using SAS software. Alpha diversity index was analyzed using one-way ANOVA and Tukey multiple comparisons to compare the differences among treatment groups. Analyses at the genus level were performed mainly on the colonies with an average relative abundance of 1% or more of the samples. $P < 0.05$ indicates significant differences, $p < 0.01$ indicates highly significant differences, and $p \geq 0.05$ indicates non-significant differences.

4. Experimental results and analysis

4.1. Changes in Alpha diversity of rumen flora in lambs

In this study, the Illumina HiSeq platform was used for 16S rDNA gene sequencing. 3,692,261 Raw PE sequences were obtained from 72 samples, and the downstream sequences were spliced, optimized, and quality-controlled, resulting in a total of 1,786,752 high-quality sequences (Clean tags). Based on the minimum number of sequences detected, 24,816 sequences per sample were taken for subsequent analysis. The goods coverage values of all the samples were above 0.98, indicating that the sequencing depth could accurately reflect the composition of rumen microorganisms in lambs. The clean tags of all the samples were clustered (or noise reduction) with 97% consistency to produce a total of 4356 OTUs, and 4274 OTUs remained after the leveling process, which means that a total of 4274 OTUs were obtained from 72 samples of rumen contents of lambs, of which 545, 714, and 890 were common to lambs in the three treatment groups at 26, 35, and 63 d, respectively, and the number of common rumen contents colonies gradually increased with the increase of age. At 26 d, the numbers of lamb-specific OTUs in CON, EW and BL groups were 316, 36 and 140, respectively, at 35 d, the numbers of lamb-specific OTUs in CON, EW and BL groups were 72, 14 and 51, respectively, and at 63 d, the numbers of lamb-specific OTUs in CON, EW and BL groups were 64, 31 and 53, respectively, which can be seen that the diversity of the rumen changes in the Alpha diversity of the rumen flora of lambs in each treatment group are shown in **Table 1**. Compared with the CON group, the richness and diversity of rumen microorganisms in lambs in the EW group were significantly lower at 26 and 35 d ($P < 0.05$), and there was no significant difference between the two groups at 63 d ($P > 0.05$). There were no significant differences in rumen flora abundance and diversity indices between lambs in the EW and BL groups at 63 d ($P > 0.05$).

Changes in the Alpha diversity of the rumen flora of lambs as a function of age are shown in **Table 2**. The results showed that the PD whole tree and Shannon index of the rumen flora of lambs in the normally weaned group (CON group) were significantly lower at 35 d ($P < 0.05$), and significantly higher and higher at 63 d than at 26 d ($P < 0.05$). Ruminal flora richness and diversity of lambs in the early weaning groups (including EW and BL groups) were highest at 63 d.

Table 1. Comparison of rumen bacterial Alpha diversity of lambs among different groups.

Items	Age	CON	EW	BL	SEM	*P*-Value
Chao1	26	1320^{ab}	1002^{c}	1246^{b}	45.24	< 0.001
	35	1270^{a}	1075^{b}	1263^{a}	33.57	0.003
	63	1362	1431	1291	28.83	0.387
Observed species	26	931^{ab}	617^{c}	780$^{b\,c}$	38.61	0.001
	35	784^{a}	655^{b}	823^{a}	25.23	0.001
	63	874	897	793	18.63	0.226

Table 1. (*Continued*).

Items	Age	CON	EW	BL	SEM	*P*-Value
PD whole tree	26	78.58[a]	55.53[b]	59.90[b]	2.85	< 0.001
	35	61.24[a]	53.07[b]	62.06[a]	1.42	0.001
	63	68.14	68.73	64.26	1.08	0.481
Shannon	26	6.34[a]	4.72[b]	4.77[b]	0.21	0.009
	35	5.38[a]	4.55[b]	5.48[a]	0.12	0.009
	63	6.02	5.66	5.57	0.11	0.302

Note: CON group (lambs were conventional weaned at 49 days of age), EW group (lambs were early weaned at 21 days of age), and BL group (lambs were supplemented with 60 mg/kg BW B. licheniformis with ≥ 4.0×10^9 cfu/g live bacteria and early weaned). Different lowercase letters in row represent significant differences ($P < 0.05$). Same lowercase letters or no letters represent no significant difference ($P > 0.05$). The same as below.

Table 2. Variation of rumen bacterial Alpha diversity of lambs among different groups.

Group	Item	d26	d35	d63	SEM	*P*-value
Conventional weaned group	Chao1	1320	1270	1362	31.5	0.514
	Observed species	931	784	874	26.5	0.066
	PD whole tree	78.6[a]	61.2[b]	68.1[b]	2.14	0.000
	Shannon	6.3[a]	5.4[b]	6.0[b]	0.15	0.013
Early weaned group	Chao1	1240[ab]	1116[b]	1352[a]	28.3	0.002
	Observed species	785[ab]	696[b]	853[a]	21.4	0.008
	PD whole tree	65.9[a]	55.2[b]	67.0[a]	1.49	0.001
	Shannon	4.9[b]	5.0[b]	5.6[a]	0.10	0.008

Note: Conventional weaned group (lambs were weaned at 49 days of age, CON group, $n = 6$), early weaned group (lambs were early weaned at 21 days of age, include EW and BL group, $n = 18$).

Table 3. Variation of rumen microbiota of the dominant genus with age of lambs.

Items	Taxa	26 d	35 d	63 d	SEM	*P*-value
Conventional weaned group	Prevotella 7	0.16[b]	7.04[a]	4.97[ab]	1.11	0.003
	Prevotella 1	25.14[a]	1.73[b]	8.50[b]	3.26	0.004
	Syntrophococcus	1.23[b]	6.77[a]	2.66[ab]	0.93	0.034
	Dialister	0.00[c]	6.14[a]	3.07[b]	0.74	0.002
	Megasphaera	0.10[b]	1.01[a]	0.96[ab]	0.17	0.006
	Ruminococcaceae UCG-014	0.25[b]	3.01[a]	1.69[ab]	0.38	0.007
	Rikenellaceae RC9 gut group	6.53[a]	0.71[b]	0.68[b]	1.05	0.042
Early weaned group	Prevotella 1	3.38[b]	0.77[b]	8.89[a]	0.96	< 0.001
	Olsenella	3.78[ab]	7.27[a]	2.33[b]	0.68	0.001
	Syntrophococcus	4.67[ab]	6.73[a]	1.56[b]	0.80	0.001
	Dialister	2.08[b]	7.18[a]	2.85[b]	0.53	< 0.001
	Roseburia	1.93[b]	0.66[b]	8.15[a]	0.88	< 0.001
	Megasphaera	2.83[ab]	4.22[a]	0.76[b]	0.4	0.000
	Ruminococcaceae UCG-014	0.16[b]	3.40[a]	1.71[ab]	0.38	< 0.001
	Selenomonas 1	2.30[a]	0.22[b]	0.29[b]	0.32	< 0.001

We identified a total of 296 genus-level groups of bacteria in the rumen contents of lambs, of which 19.13% were unclassifiable sequences, 54 group species greater than 0.1%, totaling 78.61%, and 20 group species greater than 1.0 %, totaling 66.74%, of which Erysipelotrichaceae UCG-002, Prevotella spp. 7, Prevotella spp. 1, Olsenella spp, Lachnospiraceae NK3A20 group, Lower Prevotella spp. and Mutococcus spp. were the main bacterial genus classes comprising the rumen contents of lambs. The abundance of rumen Prevotella spp. 7, Prevotella spp. 1, Mutualococcus spp., Micrococcus spp., Megacoccus spp. and Ruminalococcaceae UCG-014 in lambs of the normal weaning group changed significantly with increasing age. The proportions of rumen Prevotella spp. 1, Eubacterium spp., Reciprocococcus spp., Micrococcus spp., Rossella spp., Megacoccus spp., Ruminalococcaceae UCG-014, and Lunar Aeromonas spp. 1 in lambs of the early weaning group varied with the increase in day old (**Table 3**).

4.2. Effect of different treatments on rumen fermentation parameters in lambs

As can be seen from **Table 4**, the rumen pH of all lambs ranged from 5.42 to 6.26. Early weaning and feeding B. licheniformis had no significant effect on the rumen pH of lambs ($P > 0.05$), and the change of rumen pH of lambs was mainly affected by the age of lambs ($P > 0.05$), and the rumen pH of lambs in each treatment group at 63 d was significantly higher than that of the lambs at 26 d and 35 d ($P < 0.05$).

Table 4. Changes of rumen fermentation parameters of lambs in different groups.

Items	Age(d)	Group			SEM	*P*-value		
		CON	EW	BL		Group	Day	Group*Day
pH	26	5.67^{bc}	5.63^{bc}	5.53^{bc}	0.05	0.784	< 0.001	0.835
	35	5.52^{bc}	5.61^{bc}	5.54^{bc}				
	63	6.25^{a}	6.02^{ab}	6.26^{a}				
NH_3-N (mg/100 mL)	26	10.55^{c}	8.05	9.95^{c}	0.79	0.388	< 0.001	0.328
	35	15.74^{abc}	12.85^{bc}	11.91^{bc}				
	63	17.45^{abc}	22.84^{a}	18.56^{ab}				
TVFA (mmol/L)	26	66.44^{c}	109.64^{ab}	108.47^{ab}	3.19	0.006	0.003	0.241
	35	106.51^{ab}	121.96^{ab}	112.38^{ab}				
	63	92.94^{bc}	97.59^{b}	109.39^{ab}				
Acetate (mol/100 mol)	26	50.90	49.32	50.71	0.73	0.939	0.554	0.356
	35	50.05	55.82	52.02				
	63	52.84	50.20	54.36				
Propionate (mol/100 mol)	26	24.14	29.41	27.18	0.49	0.333	0.799	0.408
	35	26.42	27.37	25.56				
	63	24.98	26.17	24.29				
Butyrate (mol/100 mol)	26	20.12	14.81	17.43	0.82	0.449	0.103	0.864
	35	16.48	10.00	15.43				
	63	16.71	17.05	16.54				

Table 4. (*Continued*).

Items	Age(d)	Group				SEM	*P*-value		
		CON	EW	BL			Group	Day	Group*Day
Valerate (mol/100 mol)	26	1.90	4.93^{abc}	3.30^{c}		0.24	0.171	< 0.001	0.515
	35	5.34^{abc}	5.59^{abc}	5.63^{ab}					
	63	3.06	3.45^{bc}	2.62					
Isobutyrate (mol/100 mol)	26	1.00^{ab}	0.79^{b}	0.61^{b}		0.05	0.085	0.025	0.113
	35	0.82^{b}	0.55^{b}	0.63^{b}					
	63	0.91^{ab}	1.34^{a}	0.93^{ab}					
Isovalerate (mol/100 mol)	26	1.93^{a}	0.74^{bc}	0.78^{bc}		0.09	0.002	0.001	0.108
	35	0.88^{bc}	0.68^{c}	0.72^{bc}					
	63	1.51^{ab}	1.79^{a}	1.26^{abc}					
A/P	26	2.13	1.68	1.88		0.06	0.451	0.831	0.361
	35	2.05	2.08	2.11					
	63	2.27	1.94	2.27					

Lamb rumen NH3-N concentrations did not differ significantly ($P > 0.05$) between treatment groups, and all increased ($P < 0.05$) with increasing age. At 26 d, the rumen TVFA concentrations of lambs in the EW and BL groups were significantly higher than those in the CON group ($P < 0.05$), but there were no significant differences in rumen TVFA concentrations of lambs between treatment groups at 35 and 63 d ($P > 0.05$). Changes in rumen TVFA concentration were influenced by age, and rumen TVFA was significantly higher in lambs of all treatment groups at 35 d than at 26 and 63 d ($P < 0.05$), but the difference was not significant between 26 and 35 d ($P > 0.05$). Ruminal acetic acid, molar ratios of propionic, butyric, valeric, and isobutyric acids, and ethyl/propion were unaffected by treatment ($P > 0.05$), and the molar ratio of rumen isobutyric acid was significantly higher in the CON group than in the EW and BL groups ($P < 0.05$) at 26 d. There was no interaction ($P > 0.05$) between treatment and age on indicators of rumen fermentation parameters in lambs.

4.3. Changes in the Alpha diversity of lamb jejunal contents flora

A total of 1,708,416 Clean tags were obtained from 72 samples of intestinal contents, and based on the minimum number of sequences detected, 23,728 sequences per sample were taken for subsequent analysis. A total of 4381 OTUs were generated, with 4256 remaining after the draw leveling process, and species annotated to 37 phyla, 94 classes, 151 orders, 271 families and 576 genera. The total number of OTUs shared by lambs in all treatment groups was 205, 922 and 1042 at 26 d, 35 d and 63 d, respectively. At 26 d, the numbers of OTUs specific to lambs in the CON, EW and BL groups were 106, 21 and 87, respectively, at 35 d, the numbers of OTUs specific to them were 108, 50 and 66, respectively, and at 63 d, the numbers of OTUs specific to them were 53, 84 and 43, respectively, suggesting that the number of common flora in the jejunal contents of lambs gradually increased with the increase in age.

Changes in the Alpha diversity of lamb jejunal contents flora are shown in **Table 5**. As can be seen from the table, at 26 d and 35 d, there was no significant difference ($P > 0.05$) between the EW group and the CON group in terms of the abundance and diversity of the jejunal contents flora of lambs in the EW group. At 63 d, the Shannon index of the jejunal contents flora of lambs in the BL group was significantly higher than that in the EW group ($P < 0.05$).

Table 5. Comparison of Alpha diversity of lambs jejunum content microbiota among different groups.

Items	Age	CON	EW	BL	SEM	*P*-Value
Chao1	26	273	210	258	27.4	0.826
	35	1247	1083	1344	114.9	0.651
	63	1348	915	1364	109.2	0.355
Observed species	26	210	151	184	22.4	0.829
	35	741	659	828	64.0	0.599
	63	865	611	867	68.6	0.419
PD whole tree	26	21.42	16.07	16.93	1.43	0.508
	35	112.31	71.95	91.54	6.87	0.206
	63	66.00	53.44	68.24	5.66	0.444
Shannon	26	3.21	3.36	3.74	0.21	0.840
	35	5.21^{a}	4.10^{b}	5.09^{a}	0.15	0.026
	63	5.48	4.91	5.72	0.19	0.443

Note: CON group (lambs were conventional weaned at 49 days of age), EW group (lambs were early weaned at 21 days of age), and BL group (lambs were supplemented with 60 mg/kg BW B. licheniformis with $\geq 4.0 \times 10^9$ cfu/g live bacteria and early weaned). Different lowercase letters represent significant differences ($P < 0.05$). Same lowercase letters or no letters represent no significant difference ($P > 0.05$). The same as below.

Alpha diversity of the jejunal contents flora of lambs in each treatment group as affected by age. All indices of jejunal contents flora were significantly higher ($P < 0.05$) at 35 and 63 d in CON group lambs compared with those at 26 d. However, there were no significant differences in Chao1, observed species, and Shannon indices at 35 and 63 d ($P > 0.05$), and the PD whole tree index was significantly higher than that at 63 d ($P < 0.05$) (**Table 5**), indicating that the abundance and diversity of intestinal flora in CON group lambs stabilized at 35 d with increasing age. The Chao1 index, observed species and PD whole tree of jejunal contents flora of lambs in the EW group were significantly higher ($P < 0.05$) at 35 and 63 d compared with those at 26 d and reached a maximum at 35 d and decreased at 63 d, while Shannon's index increased progressively with age. This indicates that the abundance of jejunal flora of lambs in the EW group gradually increased with increasing age, and the diversity decreased at 5 d post-weaning, and then fell back after a significant increase at 14 d post-weaning, in a process of continuous adjustment and stabilization. The Chao1 index, observed species and Shannon index of jejunal contents flora of lambs in the BL group increased gradually with day old, and the PD whole tree all increased first and then decreased with day old (**Table 6**).

Table 6. Variation of jejunum content microbiota of Alpha diversity with age of lambs.

Group	Item	d26	d35	d63	SEM	*P*-value
Conventional weaned group	Chao1	276^{b}	1247^{a}	1348^{a}	159.6	0.003
	Observed species	210^{b}	741^{a}	865^{a}	92.2	0.002
	PD whole tree	21.4^{c}	112.3^{a}	66.0^{b}	11.57	0.001
	Shannon	3.2^{b}	5.2^{a}	5.5^{a}	0.31	< 0.001
Early weaned group	Chao1	229^{b}	1124^{a}	1231^{a}	86.6	< 0.001
	Observed species	167^{c}	691^{b}	796^{a}	53.0	< 0.001
	PD whole tree	16.3^{c}	82.3^{b}	67.3^{a}	4.86	< 0.001
	Shannon	3.5^{c}	4.6^{b}	5.4^{a}	0.17	< 0.001

Note: Conventional weaned group (lambs were weaned at 49 days of age, CON group, n = 6), early weaned group (lambs were early weaned at 21 days of age, included EW and BL group, n = 18). The same as below.

A total of 576 bacterial groups were annotated at the genus level, with 19.13% of the sequences being unclassifiable and 47 group species greater than 0.1%, totaling 91.37%, Olsenella, Syntrophococcus, Bifidobacterium, Ruminococcus 2, Lactobacillus and Erysipelotrichaceae UCG-002 are the main genera that make up the contents of the jejunum in lambs.

Abundance of Lactobacillus spp., Eubacterium coprostanoligenes group and Eubacterium nodatum group in jejunal contents of normal weaned lambs changed significantly with age. The abundance of Eubacterium coprostanoligenes group, Megacoccus spp, Ruminococcus gauvreauii group, Eubacterium nodatum group, Catenisphaera and Ruminalococcaceae UCG-014 in jejunal contents of lambs in the early weaning group was significantly affected by age (**Table 7**).

Table 7. The genus of bacteria of lambs jejunum content affected by age.

Items	Taxa	26 d	35 d	63 d	SEM	*P*-value
conventional weaned group	Lactobacillus	61.50^{a}	3.95^{b}	0.13^{b}	7.71	0.001
	Eubacterium coprostanoligenes group	0.72^{b}	5.17^{ab}	11.57^{a}	1.56	0.004
	Eubacterium nodatum group	0.53^{b}	3.71^{a}	1.62^{ab}	0.5	0.016
	Erysipelotrichaceae UCG-002	1.02^{b}	11.68^{a}	5.43^{ab}	1.44	< 0.001
early weaned group	Eubacterium coprostanoligenes group	0.74^{b}	4.52^{ab}	8.79^{a}	1.11	< 0.001
	Megasphaera	5.12^{a}	7.49^{a}	1.03^{b}	0.74	0.000
	Ruminococcus gauvreauii group	2.83^{ab}	1.32^{b}	6.96^{a}	0.75	0.001
	Eubacterium nodatum group	0.93^{b}	4.53^{a}	0.81^{b}	0.44	< 0.001
	Catenisphaera	0.50^{b}	1.41^{ab}	2.71^{a}	0.31	0.000
	Ruminococcaceae UCG-014	0.01^{b}	0.72^{ab}	2.45^{a}	0.35	< 0.001

4.4. Alpha diversity analysis of lamb jejunal mucosa flora

A total of 1,943,424 high-quality sequences were obtained from 72 samples of jejunal mucosa of lambs in the experiment, and 26,992 sequences were taken from each sample for subsequent analysis, which yielded a total of 4356 OTUs, with 4207 remaining after pumping and flattening process, and the species annotation yielded 24 phyla, 58 orders, 100 orders, 197 families, and 516 genera of mycobacterial flora.

The analysis showed that the total number of OTUs shared by lambs in the three treatment groups was 749, 545 and 676 at 26d, 35d and 63d, respectively. At 26 d, the number of OTUs specific to lambs in the CON, EW, and BL groups was 271, 179, and 81, respectively. At 35 d, the number of OTUs specific to lambs in the CON, EW, and BL groups was 140, 42, and 131, respectively. At 63 d, the number of OTUs specific to lambs in the CON, EW, and BL groups was 73, 208, and 115, respectively. This indicates that the number of shared jejunal mucosal flora in lambs decreased with age at 35 d and then increased at 63 d. However, the number of shared flora in lambs of all treatment groups was the highest at 26 d. Comparing the CON and EW groups individually, it can be seen that the number of OTUs common to lambs in the early weaned and normal weaned groups decreased and then increased with age, suggesting that the richness or diversity of the flora of lambs in both groups was affected by changes in age.

The results of the Alpha diversity of the jejunal mucosal flora of lambs are shown in **Table 8**. There was no significant difference in the Alpha diversity index of the jejunal mucosal flora of lambs among the groups ($P > 0.05$), and the differences in the different treatment groups with the change of age were not significant ($P > 0.05$), which indicated that early weaning or feeding additives did not have a significant effect on the abundance and diversity of the mucosal flora of the intestinal tract of the lambs.

The changes of Alpha diversity index of jejunal mucosal flora with age are shown in **Tables 3–9**. The results showed that the richness and diversity of jejunal mucosal flora of lambs in the normal weaning group decreased with age, and stabilized at 35 days. Abundance and diversity of jejunal mucosal flora in lambs of the early weaning group did not change with increasing age.

Table 8. Comparison of jejunum mucosa bacterial Alpha diversity of lambs.

Items	Age	CON	EW	BL	SEM	*P*-Value
Chao1	26	276	210	258	27.4	0.826
	35	1247	1083	1344	114.9	0.651
	63	1348	915	1364	109.2	0.355
Observed species	26	210	151	184	22.4	0.829
	35	741	659	828	64.0	0.599
	63	865	611	867	68.6	0.419
PD whole tree	26	21.42	16.07	16.93	1.43	0.508
	35	112.31	71.95	91.54	6.87	0.206
	63	66.00	53.44	68.24	5.66	0.444
Shannon	26	3.21	3.36	3.74	0.21	0.840
	35	5.21[a]	4.10[b]	5.09[a]	0.15	0.026
	63	5.48	4.91	5.72	0.19	0.443

Table 9. Variation of jejunum mucosa microbiota of Alpha diversity with age of lambs.

Group	Item	d26	d35	d63	SEM	*P*-value
Conventional weaned group	Chao1	968	781	789	38.3	0.070
	Observed species	695^{a}	509^{b}	528^{b}	28.9	0.006
	PD whole tree	177.6^{a}	80.2^{b}	57.7^{b}	19.61	0.018
	Shannon	5.8	5.7	6.0	0.20	0.873
Early weaned group	Chao1	814	727	827	32.4	0.398
	Observed species	555	487	573	22.8	0.273
	PD whole tree	94.8	78.6	105.4	9.50	0.520
	Shannon	5.3	5.2	5.9	0.18	0.265

Note: Conventional weaned group (lambs were weaned at 49 days of age, CON group, n = 6), early weaned group (lambs were early weaned at 21 days of age, included EW and BL group, n = 18).

The relative abundance of Lactobacillus spp, Eubacterium coprostanoligenes group, Bacteroides spp, Megacoccus spp, Rikenellaceae RC9 gut group and Christensenellaceae R-7 group in the jejunal mucosa of lambs in the normal weaning group changed significantly with the increase in age, Early weaning group of lambs jejunal mucosa Eubacterium coprostanoligenes group, Bacteroidetes spp, Blautia, Eubacterium nodatum group, Ruminococcaceae UCG-014, Bacteroidetes spp, Megacoccus spp, Faecalibacterium, Mitsuokella, and Christensenellaceae R-7 group changed in relative abundance with age (**Table 10**).

Table 10. The genus of bacteria of lambs jejunum mucosa affected by age.

Items	Taxa	26 d	35 d	63 d	SEM	*P*-value
Conventional weaned group	Lactobacillus	26.35^{a}	4.82^{ab}	0.69^{b}	3.86	0.013
	Eubacterium nodatum group	0.52^{b}	5.13^{a}	1.16^{ab}	0.82	0.020
	Dialister	0.02^{b}	4.22^{a}	0.92^{b}	0.58	0.003
	Megasphaera	0.13^{b}	2.60^{a}	0.27^{b}	0.42	0.009
	Rikenellaceae RC9 gut group	0.09^{b}	0.21^{b}	3.26^{a}	0.57	0.020
	Christensenellaceae R-7 group	2.51^{a}	0.21^{b}	1.35^{ab}	0.37	0.034
Early weaned group	Eubacterium coprostanoligenes group	0.39^{b}	4.65^{ab}	8.72^{a}	1.18	< 0.001
	Bacteroides	6.77^{a}	2.20^{b}	1.42^{b}	0.77	0.018
	Blautia	4.36^{a}	2.01^{b}	1.38^{b}	0.34	0.006
	Eubacterium nodatum group	1.26^{b}	6.20^{a}	0.67^{b}	0.63	< 0.001
	Ruminococcaceae UCG-014	0.68^{b}	1.83^{ab}	3.98^{a}	0.47	0.002
	Dialister	0.33^{b}	4.11^{a}	1.37^{b}	0.38	< 0.001
	Megasphaera	0.57^{b}	4.86^{a}	1.10^{b}	0.54	< 0.001
	Faecalibacterium	0.85^{b}	0.94^{b}	2.50^{a}	0.27	0.004
	Mitsuokella	0.52^{b}	3.52^{a}	0.30^{b}	0.43	< 0.001
	Christensenellaceae R-7 group	2.54^{a}	0.29^{b}	0.97^{ab}	0.36	0.001

5. Discussion

Ruminal VFA concentration increases with solid feed intake and is influenced by dietary (e.g., nutrient level, composition) or animal factors (e.g., rate of

absorption, etc.). Ruminal development is not only stimulated by VFA, but also by nutrients provided by liquid feeds [18]. In the present study, rumen pH and NH3-N concentration of lambs in each treatment group were at a steady state. Lambs were weaned at 21 d, and the high intake of solid feed after weaning promoted the development of the rumen in lambs, which is consistent with previous studies that have concluded that early provision of solid feed facilitates the rapid development of the rumen [19]. In the present study, rumen TVFA concentrations of lambs in the early weaned EW and BL groups were significantly higher than those in the CON group at 5 days after weaning (26 days of age), and there was no significant difference at 35 and 63 d. This is consistent with Yang et al.'s [20] study that concluded that rumen TVFA concentrations of lambs changed before and after weaning. This is because after early weaning of lambs, there is a sudden change from feeding on breast milk to feeding on kibble, and solid feed intake rises rapidly, which can provide more substrate for rumen fermentation in lambs, whereas lambs in the CON group were mainly fed on breast milk as the main nutrient, so that there was a large difference in rumen TVFA at 26 days of age. In contrast, at 35 and 63 days of age, rumen fermentation in the CON group was essentially identical to that in early weaned EW and BL lambs as the amount of open feed taken by the CON group continued to increase. This may be related to the change in lamb diets, as lamb starters did not contain roughage between 7 and 35 d, and lamb starters were changed to starters containing 12% roughage and higher levels of dietary NDFs from 36 to 63 d, which may be the reason for the increase in rumen pH and the decrease in TVFA concentration at 63 d.

After ruminants are born, various types of microorganisms quickly colonize the rumen, and the type of colonization is influenced by many factors, such as exposure to adult animals, initial solid feed offered, and feed composition [21]. Our results showed that the abundance and diversity and composition of rumen microorganisms in lambs changed with the onset of weaning, and their effects were mainly at 5 d post-weaning, with higher similarity in the rumen flora of lambs at 14 and 42 d post-weaning, and the early effects had less of a sustained effect in the later stages of the process, and also from the rumen fermentation parameters of the lambs, which showed that the rumen VFA concentration of the lambs changed mainly at 5 d post-weaning. Studies have shown that the early stages of young animals may be a window for regulating the colonization of the rumen microbiota, and that nutritional interventions at an early stage of life may have an impact on the composition of the rumen flora [9]. This experimental study showed that early feeding of B. licheniformis increased the diversity of rumen flora in lambs at 26 d, contributing to an early rise in rumen flora diversity.

In the present study, differences in flora composition at the genus taxonomic level were found between early weaned and normally weaned lambs. At 5 d after early weaning, the relative abundance of Prevotella spp. 1 in the rumen of lambs was significantly lower in the early weaned group compared with the control group, and the relative abundance of Prevotella spp. 7, Lower Prevotella spp., Reciprocococcus spp., Bacteroidetes spp. and Megalococcus spp. was significantly higher. Prevotella spp. is the dominant genus in the developing rumen and is the most abundant and

prevalent group of rumen microorganisms, degrading and utilizing starch and plant cell wall polysaccharides such as xylan and pectin in the rumen, with the fermentation products being acetic, succinic, and propionic acids, etc. The decrease in the relative abundance of this group was accompanied by an increase in the number of feed fiber-digesting bacteria such as Reciprocal coccus spp. and small class of bacilli spp., suggesting a significant increase in solid feed intake in lambs after early weaning, whereas control lambs still consumed less open feed at this time, and an increase in fiber-digesting flora, suggesting that the rumen microbiota of lambs continues to mature [22].

Early colonization of the intestinal flora plays an important role in contributing to host health by facilitating the establishment of intestinal barrier function and the maturation of the immune system [23] and in maintaining intestinal epithelial integrity. In contrast to the stable flora of adult animals, the intestinal flora is highly unstable at a young age. The diversity of the flora in the jejunum of lambs changes with increasing day old. Early weaning reduced the diversity of jejunal flora in lambs in all comparisons at 26, 35 and 63 d, respectively. It showed that early weaning had a certain effect on the jejunal flora of lambs in the short term, and the effect lasted until 63 d. In the normal weaning group, the diversity of jejunal flora increased with the increase of day old, and then stabilized at 35 d. In the early weaning group, the diversity of jejunal flora was lower at 5 d after weaning, and then increased at 14 d after weaning, and then decreased, which was in the process of continuous adjustment and stabilization.

Numerous studies have shown that the intestinal flora of animals is extremely unstable after birth, and early intervention of the intestinal flora is a critical window for optimizing the immunity of individual animals [24,25]. Probiotic supplementation can increase beneficial intestinal flora while inhibiting potential pathogens, boosting certain immune factors and reducing inflammation levels, and beneficial microorganisms can enhance intestinal function by producing certain metabolites such as short-chain fatty acids.

6. Conclusion

In summary, early weaning did not affect final flora abundance and diversity, although it resulted in a short-term decrease in the abundance and diversity of the rumen and jejunal contents and mucosal flora of the lambs, which adjusted in advance to a steady state [26]. Early feeding of B. licheniformis helps lambs to rapidly increase the abundance and diversity of flora after weaning stress. Changes in rumen and intestinal flora are adaptations to feed and environmental changes, and 35 d is the point at which weaning stress is recovered and gastrointestinal flora stabilization is established. Dietary changes brought about by lamb age and weaning are major determinants of rumen microbiota and fermentation parameters. The high intake of solid feed after weaning promoted the development of the rumen in lambs, and early feeding of B. licheniformis had no significant effect on rumen fermentation parameters in lambs.

Author contributions: Conceptualization, SL and ZH; methodology, SL; software, ZH; validation, SL and ZH; formal analysis, SL; investigation, SL; resources, SL; data curation, SL; writing—original draft preparation, SL; writing—review and editing, ZH; visualization, SL; supervision, ZH; project administration, SL; funding acquisition, SL. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: I would like to express my sincere gratitude to all those who helped me in the completion of this study. Special thanks go to my supervisor, from the selection of the thesis topic, the research design to the final draft, every step of the process has gathered the efforts and wisdom of my supervisor. Also, I would like to thank my classmates and friends. In the process of thesis writing, we learn from each other and made progress together. I would also like to thank the staff of the library, provided the convenient conditions and quality services, which created a favourable environment for my research work.

Ethical approval: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of TOPBIOTECH IACUC (protocol code TOP-IACUC-2023-0221, and date of approval 2023-9-1).

Informed consent: Not applicable.

Conflict of interest: The authors declare no conflict of interest.

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