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Full Length Article

Effect of *Boulardii* Yeast Wall Polysaccharides on Intestinal Microflora in Jejunum, Cecum and Colon for Early-Weaned Lambs by 16S rRNA Sequence Analysis

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Abstract

This study was conducted to investigate the effects of *Boulardii* yeast wall polysaccharide (BRYP) on the intestinal micro-ecosystem of early-weaned lambs. A total of 60 early-weaned lambs (35-days-old, Kazak♂*Altay♀*Suffolk♂) were randomly assigned into two treatments: a control group with a basal diet, an experimental group with a basal diet and added 0.05% BRYP. The HiSeq high-throughput sequencing analysis of 16S rRNA was used to investigate the differences in intestinal microbial flora composition, diversity, relative abundance, principle coordination analysis, and the correlation between intestinal microbial flora composition and immune indices. After feeding for 40 days, the addition of 0.5% BRYP in milk replacer significantly enhanced the species richness in the cecum and colon, but decreased the diversity of species in the colon ($P < 0.05$); Compared with the control group, the relative abundance of *Bacteroidetes* in the experimental group was significantly enhanced, but the *Proteobacteria* was significantly decreased in all tested intestinal segments ($P < 0.05$). In the jejunum, the relative abundance of *Lactobacillus*, *Prevotella*, and *Fibrobacter* of the experimental group were significantly enhanced than that of the control group, but the *Ruminobacter* was significantly decreased ($P < 0.05$); In the cecum, the relative abundance of *Bacteroides*, *Lactobacillus*, *Oscillospira* and *Bifidobacterium* of the experimental group were significantly enhanced than that of the control group, but the *Blautia* were significantly decreased ($P < 0.05$); In the colon, the relative abundance of *Akkermansia, Bifidobacterium, Lactobacillus* and *Faecalibacterium* of the experimental group were significantly enhanced than that of the control group, but the *Prevotella*, *Streptococcus*, and *Escherichia* were significantly decreased ($P < 0.05$). There were significant correlations between intestinal immune indices (IL-6, IL-10, TNF-α) and intestinal microbial composition in the colon ($P < 0.05$). These results indicated that BRYP may contribute to the promotion of the proportion of helpful microbial populations and enhancing the balance of intestinal; Besides, BRYP may indirectly improve the intestinal immune function by changes of intestinal microflora composition, but suppress the inflammatory response in the bottom of intestinal mucosa of early-weaned lambs.

Keywords: *Boulardii* yeast wall polysaccharides; 16S rRNA sequence; Intestinal microflora; Immunity indices; Correlation analysis; Early-weaned lambs

Introduction

Lambs intestinal mucosa is in continual contact with diverse array of microbes. The intestinal microflora is vital to physiological functions and homeostatic, and plays important roles in early-weaned lamb's defense through colonization resistance by promoting the development and regulation of the acquired mucosal immune system (McCoard *et al.* 2019). Disturbances of intestinal microflora homeostasis are thought to contribute to severe gastrointestinal disorders (Kong *et al.* 2019). The intestinal microflora comprises potential pathogens and can be a source of infection under some circumstances (Yang *et al.* 2019a). Especially for early-weaned lambs, the immune system and digestive were immature, and they had to adapt the radical change from digestible watery breast milk to a relatively indigestible solid feed, and the risks of infection by pathogenic bacteria such as *Escherichia coli* and *Salmonella* were increased (Fan *et al.* 2019.)

Boulardii yeast wall polysaccharide (BRYP) is one of the main biologically active components in *Boulardii* yeast. Besides, BRYP is a kind of natural prebiotics, which are compounded from *β*-glucan (30–60%), mannosan (20–30%), and chitin (5–10%) (Chen *et al.* 2018a). BRYP almost does not be digested and absorbed by the early-weaned host digestive system, but could increase the proliferation of kinds of probiotics selectively (Fortin *et al.* 2017).

In recent years, many studies have shown that yeast

wall polysaccharides supplementation in basal diet can regulate the balance of intestinal microbiota, and reduce the diarrheal diseases, which has caused by an imbalance of intestinal microflora and bacterial translocation. It has been reported that the yeast cell wall polysaccharides supplementation could improve the growth performance of cattle by promoting the fibrotic microbial populations, but decrease aerobic starch-utilizing bacteria (Peng *et al.* 2020). Besides, in the cecum of weaned piglets, feeding diets with yeast cell wall polysaccharides in a short feeding-period could improve the cecum bacteria structure, and enhance the advantage bacterium, such as *Firmicutes*, *Ruminococcus* (Qin *et al.* 2017). Furthermore, the relative abundance of *Bacteroidetes* and *Firmicutes* was significantly improved in the rate intestine by feeding a diet with yeast cell wall polysaccharides (Radhika *et al.* 2019).

However, little study has analyzed the effects of BRYP on the intestinal microflora of early-weaned lambs according to a HiSeq of 16S rRNA gene. Differences in intestinal microflora compositions between jejunum, cecum, and colon in early-weaned lambs after feeding BRYP are still unknown. Therefore, more pieces of evidence are needed to specify the roles of BRYP of intestinal microflora and correlation with immune indices of early-weaned lambs. Therefore, we performed 16S rRNA HiSeq to specify and compare the intestinal microbial flora composition, diversity, relative abundance, principle coordination analysis, and correlations with intestinal immune indices. We hypothesized that a supplement of 0.5% BRYP with basal milk replacer can modulate the intestinal microflora compositions. Therefore, this research might provide fundamental information for a large-scale in lamb industry.

Materials and Methods

All experimental lambs in this research have been prospectively approved and granted a formal waiver of ethics approval by the Animal Welfare Committee of Shihezi University (Xinjiang, China) with the ethical code: A2019-156-01. The experimental period was 40 days from 5th December 2019 to 18th January 2020. The experiment was conducted in Asar farming cooperatives, Changji, China (43°91’22.01” S, 87°09’93.84” W).

Materials

The Boulardii yeast was obtained from Shihezi University (Shihezi, China). The pure BRYP was obtained by the procedures of fermentation, extraction, and purification. The pure BRYP of nutritional compositions were polysaccharides (≥ 84%), crude protein (≤ 4%), crude fat (≤ 3%), crude ash (≤ 2%), and moisture (≤ 7%). The main components of BRYP were BLC-1 and BLC-2, which molecular weight were 22.76 kDa and 9.09 kDa. The BLC-1 and BLC-2 accounted for 63.12 and 28.70% in BRYP.

The trace mineral premix was provided by Tianjin Zhengda biological technology Ltd., Tianjin, China; The Baby Formula Milk Powder was provided by Bright Dairy Co., Ltd, Shanghai, China; The enzyme-linked immunosorbent assay kit was provided by Suzhou Eisai Pharmaceutical Co., Ltd, Suzhou, China; DNA isolation kit was provided by Tiangen Biochemical Technology Co., Ltd, Beijing China.

Experimental animals and experimental design

A total of sixty early-weaned lambs (35-days-old, Kazak♂*Altay♀*Suffolk♂) were randomly blocked to two groups with three replicates each and ten lambs (six males and four females per replicate pen) in each treatment. The feeding trial lasted 40 days. All lambs were enforcedly weaned at 35 old-days, and housed in thermostatically controlled livestock pens (3.0×2.5 m) which were equipped with heating facilities (maintained at 22–25°C inside) and straw paillasses (thickness 2 cm), and with sufficient warm water (15–20°C). The pens were cleaned and sterilize at the beginning of the experiment day basis to prevent disease outbreaks. Milk replacer and warm water were available ad libitum in the whole feeding trial. Feeding and vaccination procedures followed as farm management schedules. The milk replacer was mixed with warm water (50°C) as the ratio (milk replacer: water = 1:4), and 4 feeding times a day at 8:00, 13:00, 18:00, and 23:00. The control group was fed the basal milk replacer with no addiction, a formulation consisting of cottonseed meal, soybean meal, bran, and corn, which was formulated to meet the nutrient requirement of 8~25 kg lambs with the daily gain of 300 g/day recommended by the Chinese Feeding Standard of Lamb (2017) (Table 1). The experimental group was fed the basal milk replacer with 0.5% BRYP.

Sample collection and preparation

At the end of the experiment, three lambs in each replicate were randomly selected and anesthetized by injecting a 5% Nembutal solution. After 25 min of injection, the lambs were slaughtered by cutting neck vein. After lambs were dissected, the contents of the jejunum, cecum, and colon were obtained by manual extrusion, respectively, for the intestinal microflora analysis. After contents collection, the segments of jejunum, cecum, and colon were cut opened longitudinally and washed three times by 0.9% sodium chloride to remove impurities, respectively. All samples were placed immediately into sterile plastic tubes, separately, for the immune indices detection (Xu *et al.* 2016). For reducing variations between individuals, the contents samples from jejunum, cecum, and colon segment samples of three lambs were pooled into one biological sample in each treatment (Bukin *et al.* 2019). Overall, 18 samples (jejunum, cecum, and colon) were used for 16S rRNA high-throughput sequencing, and 6 samples (colon) were used for immune indices detection. All samples were fast-frozen in liquid

nitrogen and stored at -80°C until the following analysis.

DNA extraction

Total intestinal microbial genomic DNA were isolated from each gut segment by using DNA isolation kit and stored at −25°C for the following analysis.

16S rDNA amplicon pyrosequencing

PCR amplification of the 16S rDNA (V3-V4 region) was performed using the forward primer 314 F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the reverse primer 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR conditions were as follows: initial denaturation at 92°C for 5 min, 28 cycles of 92°C for 55 s, 50°C for 35 s and 70°C for 2 min, and then final extension at 70°C for 10 min (Xiao *et al.* 2017). The quality and quantity of amplified DNA were showed by agarose gel electrophoresis, separately.

The 16S rRNA gene, which has been amplified, was obtained through DNA gel extraction kit, and analysis with 1% Sepharose, which contains 2% of polyvinylpyrrolidone agarose gel electrophoresis for the quality and quantity of amplified DNA sequence (Qian *et al.* 2018). The total 16S rRNA genes were sent to Shanghai Jingjie Biomedical Technology Co., Ltd. (Shanghai, China) and to analysis microbiota by MiSeq PE300 sequencing platform. The raw data were matched by the FLASH analysis tool (Version 1.2.5, http://ccb.jhu.edu/software/FLASH/), and filtered to remove the barcode sequences, forward and reverse primers with the QIIME (Version 1.1.6, http://qiime.org/scripts/split_libraries_fastq.html) (Xiao *et al.* 2018). The read was removed if the quality scores less than 20 at the sliding window or tags contained ambiguous bases. By using the UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html), the reads were compared with the annotated database of the species, and made sure that the chimeric sequences were discarded totally (Amato *et al.* 2013).

Analysis of OTU cluster

The optimized sequences were eliminated and demultiplexed, after removing the chimeric sequences, barcode, and primers. The operational taxonomic units (OTUs) were selected (97% similarity) and clustered by using the Usearch (Version 7.0, http://drive5. com/uparse/) (Han *et al.* 2019). The most frequent sequences in OTUs were selected, and as the representative OTU sequences, which were analyzed and annotated with the method of SILVA SSU rRNA database and the mothur (threshold range from 0.6 to 1, http://www.arb-silva.de/).

Sequence analysis

We used the QIIME and R packages to mainly perform the analyses of the sequence data.

1) OUT-ranked abundance curves were conducted to compare the uniformity and richness of OTUs among samples. Venn diagram was showed to visualize the unique and shared OTUs among samples. The significance of differentiation of microflora composition among groups was analyzed by permutational multivariate assessment of variance and similarities by R package “vegan” (Lozupone and Knight 2005).

2) Alpha diversity index revealed the richness and uniformity of the microbial community. OTU alpha diversity indices, including Abundance-based Coverage Estimator (ACE), richness estimator (Chao), Simpson index, and Shannon diversity index, were calculated by the OTU table in QIIME (Schloss *et al.* 2009).

3) The OTU taxonomic compensation was operated by using BLAST searching the representative sequences set against the NCBI 16S rRNA database, and then, an OTU table was clustered to calculate the abundance of each OTU in every sample and taxonomy of those OTUs (Quast *et al.* 2012).

4) Beta diversity calculation was implemented to analyze the composition variation of microbial communities in different samples by using Principal component analysis (PCA). The difference of microbial samples was reflected in a three-dimensional coordinate diagram, which the more semblable sample composition was, the nearer the distance in PCA diagram. Linear discriminant analyses (LDAs) were used to analyze whether it was important microbial community that contributed to differences (Knights *et al.* 2011).

The correlation analysis between intestinal immune indices and intestinal bacteria was conducted by R package vegan. The heatmap diagrams were generated, according to the Spearman correlation coefficients between altered intestinal bacteria and immune indices (Akond *et al.* 2018).

Determination of immune indices

After the colon segment samples defrosting, 1 g colon segment was weighed accurately and ground with liquid nitrogen in sterile mortars for 15 min. The colon segment powder was mixed with 10 mL, 0.9% sodium chloride solution, and centrifuged at 3500 r/min, 6°C for 20 min. After that, the tissue fragments were removed, and the supernatant was collected. The levels of SIgA, IL-6, IL-10, and TNF-α in the colon segment were determined according to the instruction of ELISA kits (Wang *et al.* 2018).

Statistical analysis

The results were shown as mean ± standard deviation, and categorical variables were shown as proportions and numbers. We used unpaired Student t-test and ANOVA to statistically evaluate differences among groups, respectively. It was considered statistically significant when $P < 0.05$. All

analyses were counted by Excel (version 2017 for Windows, Microsoft Inc., Chicago, IL, USA), and represented with software SPSS (version 18, SPSS Inc., Chicago, IL, USA).

Results

Assessment of 16S rDNA PCR and sequencing

The 16S rRNA genes in each intestinal segment contents were amplified by using forward primer 314 F and reverse primer 806 R. As shown in Fig. 1, the concentrations and sizes of electrophoretic bands in the PCR electrophoresis graphic were clear and suitable. As shown in Fig. 2, the Shannon curves and Rarefaction curves have reached the platform, and Good coverage index achieved between 0.992 and 0.997, according to the analysis of software Mothur. The results represented that the vast majority of the microbial diversity information has been obtained by the samples of amplified 16S rRNA genes, and the sequencing depth was adequate in this study.

Operational taxonomic unit partition and classification

After the quality count, 902,549 high-quality sequences were acquired. On average, 50,141 ± 3,483 sequences were obtained per sample. A total of 3,678 OTUs were identified from all samples. On average, 204 OTUs were obtained per sample of the control group and experimental group, based on 97% species similarity. All detected OTUs were from 16 phyla, 29 class, 63 order, 108 families, and 287 genera. On average, there were 369, 537 and 885 OTUs in the jejunum, cecum, and colon from the control group, respectively. There were 378, 552 and 987 OTUs in the jejunum, cecum, and colon from the experimental group, respectively. Venn figures of unique and common OTUs were shown in Fig. 3. There were 261, 378 and 657 common OTUs between the control group and experimental group of jejunum, cecum, and colon, respectively. In the control group, 108, 159 and 198 unique OTUs were detected in the jejunum, cecum, and colon, respectively; whereas 117, 174 and 330 unique OTUs were detected in the jejunum, cecum, and colon of the experimental group, respectively.

Analysis of Alpha diversity

Alpha-diversity was analyzed by richness indices (Chao and ACE), and diversity indices (Shannon and Simpson). According to the results as shown in Fig. 4, firstly, we found that the richness index of Chao and ACE increased as digestive tract from top and bottom; whereas the diversity index of Shannon and Simpson firstly increased, and then decreased as digestive tract from top and bottom. Besides, the experimental group of Chao was significantly higher than the control group in the cecum ($P < 0.05$). Furthermore, the experimental group of Chao was extremely significantly higher than the control group in the colon ($P < 0.01$). The experimental group of ACE was higher than the control group in the jejunum, cecum and, colon, respectively, but the differences was not significant ($P > 0.05$). The experimental group of Shannon and Simpson were significantly lower than the control group in the colon ($P < 0.05$). These results indicated that the addition of 0.5% BRYP in basal milk replacer can enhance the richness of microbial composition in the cecum and colon, but decrease diversity in the colon.

Analysis of taxonomic composition

The taxonomic bar represented relative abundance levels of various phyla and genus in two groups. After the optimized sequences were clustered and annotated, the top-ranking most abundant microbial at phylum and genus levels were shown in Fig. 5–6. Otherwise, the sequences which could not be clustered into any groups (97% similarity) in the database were assigned as "other".

At the level of phyla, based on the average relative abundance analysis, the results indicated that the proportion of relative abundance of two major phyla (*Firmicutes* and *Bacteroidetes*) were more than 70% in all OTUs. Besides, *Proteobacteria*, *Verrucomicrobia*, *Spirochaetes*, *Fibrobacter*es, *Tenericutes*, *Lentisphaerae*, and *Euryarchaeota* were another 8 predominant phyla. In the jejunum, the experimental group of relative abundance of *Bacteroidetes* and *Verrucomicrobia* was significantly higher than the control group by 27.70 and 75.75% ($P < 0.05$), but the experimental group of relative abundance of *Proteobacteria* was significantly lower than the control group by 92.15% ($P < 0.05$). Compared with the control group, and the experimental group of relative abundance of *Bacteroidetes* in the cecum and colon was significantly increased by 30.06 and 45.89% ($P < 0.05$), but the experimental group of relative abundance of *Firmicutes* and *Proteobacteria* were significantly decreased by 7.45, 21.19 49.04 and 48.16% in the cecum and colon, separately ($P < 0.05$).

At the level of the genus, the predominant genus was *Lactobacillus*, *Prevotella*, *Ruminobacter*, *Bacteroides*, *Blautia*, *Streptococcus*, *Faecalibacterium*, *Akkermansia*, and *Bifidobacterium*. In the jejunum, the experimental group of relative abundance of *Lactobacillus*, *Prevotella*, and *Fibrobacter* was significantly higher than the control group by 48.32, 36.87 and 75.42% ($P < 0.05$), but the experimental group of relative abundance of *Ruminobacter* was significantly lower than the control group by 27.83% ($P < 0.05$). In the cecum, the experimental group of relative abundance of *Lactobacillus*, *Bacteroides*, *Oscillospira*, and *Bifidobacterium* were significantly higher than the control group by 37.65, 32.02, 86.49 and 56.52% ($P < 0.05$), but the experimental group of relative abundance of *Blautia* was significantly lower than the control group by 68.19% ($P < 0.05$). In the colon, the experimental group of relative abundance of *Lactobacillus*, *Faecalibacterium*,

Table 1: Ingredient compositions and chemical analysis of milk replacer (%; air-dry basis)

Items	Content	Nutrient levels	Content
Ingredients		Nutrition level	
Expended corn	41.50	Dry matter	90.67
Alfalfa hay	8.00	Digestible energy DE/(MJ/kg）	17.38
Soybean oil	2.00	Crude protein	23.75
Expended soy	16.00	Crude fat	16.08
Fermented Soybean	20.00	Neutral detergent fiber	4.81
Premix	1.00	Calcium	0.53
Baby Formula Milk Powder	10.00	Phosphorus	0.41
NaCl	0.30	Lysine	0.91
$CaHCO_3$	1.00	Methionine + cysteine	0.61
$NaHCO_3$	0.20	Threonine	0.65
Total	100.00	Concentrate: roughage	80:20

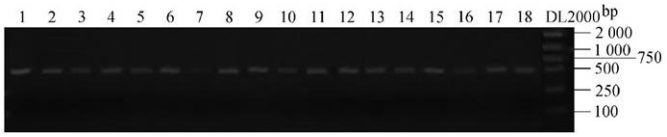

Fig. 1: The electrophoresis results of PCR products

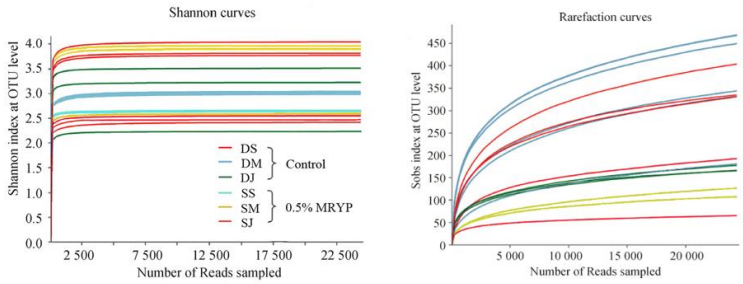

Fig. 2: Analysis results of Shannon curves and Rarefaction curves

Fig. 3: Veen diagram of common Operational Taxonomic Units (OTUs). DS,DM and DJ represent samples of jejunum, cecum and colon in the control group, respectively; SS, SM, SJ represents sample of jejunum, cecum and colon in the experimental group, respectively

Bifidobacterium, and *Akkermansia* were significantly higher than the control group by 32.25, 27.72, 72.38 and 73.21% ($P < 0.05$), but the experimental group of relative abundance of *Escherichia*, *Prevotella* and *Streptococcus* were significantly lower than the control group by 42.96, 38.27 and 30.77% ($P < 0.05$).

Analysis of Beta diversity

The Unweighted UniFrac distances of samples under the jejunum, cecum, and colon in two groups were calculated based on the relative abundances of OTUs in the eighteen samples. Besides, Principal coordinates analysis (PCoA), which based on the Unweighted UniFrac, revealed that whether there were clear separations of the microflora composition of the control group from the experimental group. The three-dimensional graph of PCoA at genus level was shown in Fig. 7. The microbial communities of the control group and the experimental group were hard to separation in the jejunum, but there was a clear separation of the microbial communities of the control group from the experimental group. This result indicated that BRYP may mainly affect the microbial communities of the bottom of the digestive tract.

To further identify specific microbial composition with statistically significant differences among groups, we used linear discriminant analysis (LDA) to analyze the specific microbial composition, which was altered by the addition of 0.5% BRYP in the basal milk replacer, as shown in Fig. 8. When taxa with LDA scores greater than 2, the experimental group of differential OTUs of jejunum, cecum and colon were 10, 11 and 17, respectively; the control group of

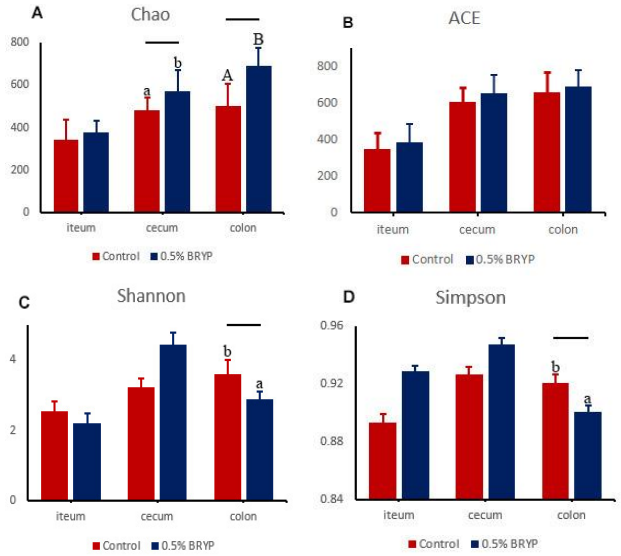

Fig. 4: Diversities of microflora composition between the control group and the experimental group in different intestinal segments. Four species richness and diversity estimators, including (**A**) Chao, (**B**) ACE, (**C**) Shannon's diversity index and (**D**) Simpson's diversity index. Different capital letters indicate extremely significant difference ($P<0.01$), and different small letters indicate significant difference ($P<0.05$)

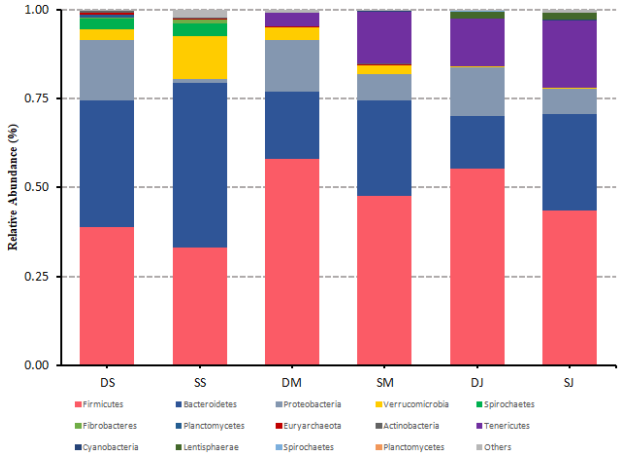

Fig. 5: Relative abundances of microbes in each intestinal segment at phylum level

differential OTUs of jejunum, cecum, and colon were 3, 11 and 14, respectively. When taxa with LDA scores greater than 3, the experimental group of differential OTUs of jejunum, cecum, and colon were 1, 4, and 9, respectively; the control group of differential OTUs of jejunum, cecum, and colon were 0, 4 and 3, respectively.

The canonical correlation analysis between immune indices and intestinal microflora

The results of the significant differences of immune indices

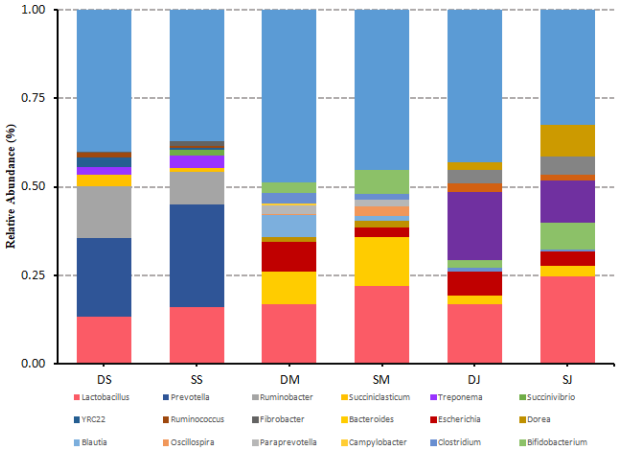

Fig. 6: Relative abundances of microbes in each intestinal segment at genus level

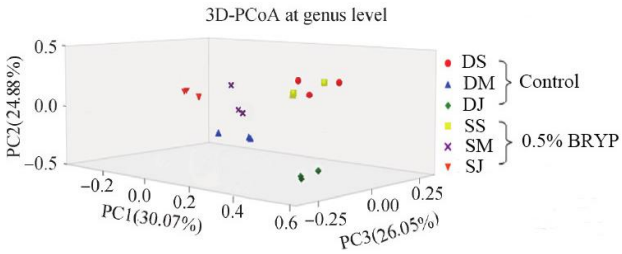

Fig. 7: The total Principle Coordination Analysis scores plot about the microbes in intestinal segments. DS,DM and DJ represent samples of jejunum, cecum and colon in the control group, respectively; SS,SM,SJ represents sample of jejunum, cecum and colon in the experimental group, respectively

and intestinal microflora were in two groups. The densities of immunoglobulins and cytokines of intestinal mucosal were analyzed according to the instruction of ELISA kits. As shown in Table 2, there was a significant difference in the two groups concerning the content of IL-6, IL-10, SIgA, and TNF-α. The experimental group of IL-6 was significantly lower than the control group by 15.09% In the colon ($P < 0.05$); The experimental group of IL-10 were significantly higher than the control group by 15.80 and 6.60% In the cecum and colon, respectively ($P < 0.05$); The experimental group of SIgA was significantly higher than the control group by 10.98% in the colon ($P < 0.05$); The experimental group of TNF-α was significantly lower than the control group by 7.90% in the colon ($P < 0.05$).

Besides, according to the analysis of taxonomic composition at the level of genus, the significant difference of top-ranking most abundant intestinal microflora was listed in Fig. 9.

The canonical correlation analysis between immune indices and intestinal bacteria in the colon as shown in Fig. 9, the clustering correlations between the compound BRYP-stimulated significant changes in immune indices (immunoglobulins and cytokines in intestinal mucosa) and

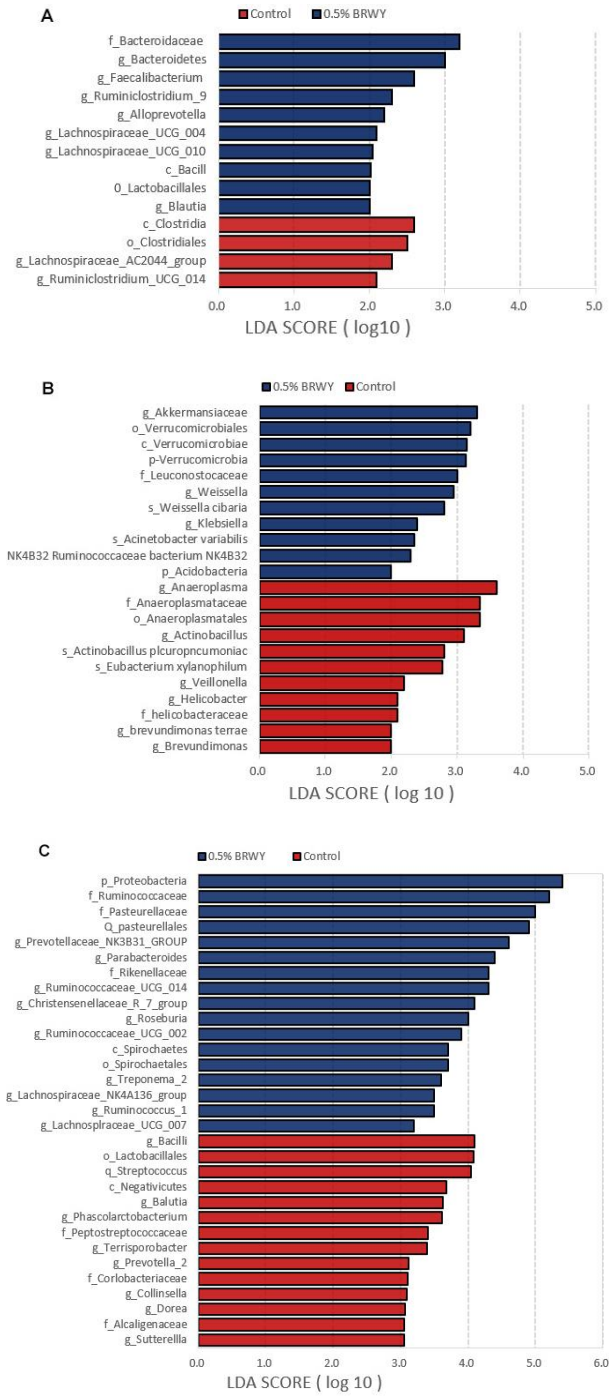

Fig. 8: The comparisons of microflora composition between the control group and the experimental group in different intestinal segments. (**A**) Comparison of microbial composition in jejunum; (**B**) Comparison of microbial composition in the cecum; (**C**) Comparison of microbial composition in the colon

intestinal bacteria relative abundance at the genus levels were analyzed to investigate the interactions between immune indices and intestinal bacteria during the feeding trial of addition BRYP-treated early-weaned lambs. The results indicated that robust correlations with compound BRYP-induced alterations in intestinal microflora and immune indices. The red bars represented positive correlations, and the blue bars indicated negative correlations.

To further predict interactions between intestinal bacteria and immune indices, according to Pearson correlation coefficients, we constructed a diagram of Canonical Correlation Analysis (CCA) between intestinal bacteria and immune indices, as shown in Fig. 10.

Combined with the heat map and CCA diagram, we summarized that there were significant correlations between IL–6, IL–10, TNF-α, and intestinal microbial composition in the colon ($P < 0.05$). IL–6 was positively correlated with *Butyricicoccus*, *Clostridiales*, *Escherichia-Shigella*, Achinobacillus, Rikenellaceae_RC9_gut_group, Norank_f_Erysipelotrichaceae, Clostridium, Prevotella_9, and *Ruminococcaceae_UCG-014*, respectively; but the negative correlations with IL–6 were *Akkermansia*, *Lactobacillus*, *Rombsia*, *Oscillospira*, Prevotella_1, Prevotellaceae_UCG-004, Lachnospiraceae_136_group, Parabacteroides, Blautia, and Lachnospiraceae NK4A136. IL-10 was positively correlated with *Bifidobacterium*, *Akkermansia*, *Lactobacillus*, *Prevotellaceae_UCG-003*, *Faecalibacterium*, *Rombsia*, *Prevotellaceae_NK3B31_group*, *Christensenellaceae_R-7group*, *Lachnospiraceae_XPB1014_group*, *Prevotellaceae_UCG-001*, and *Ruminococcaceae_UCG-005*, respectively; but the negative correlations with IL-10 were *Clostridiales*, *Escherichia-Shigella*, *Coprococcus_3*, *clostridium_sensu_stricto_1*, *Ruminococcus_1*, and *Intestinibacter*. TNF-α was positively correlated with *Streptococcus*, *Erysipelotrichaceae_UCG-003*, *Norank_f_Erysipelotrichaceae*, *Escherichia-Shigella*, *Clostridium_sensu_stricto_1*, *Prevotella_2*, and *Eggerthellaceae_unclassified*, respectively; but the negative correlations with TNF-α were *Akkermansia*, *Romboutsia*, *Christensenellaceae R-7 group*, and *Ruminococcaceae_UCG-002*.

Discussion

The intestinal microflora is a vital and complex ecosystem with functions that shape animal health. Currently, the intestinal microbial ecosystem has been regarded as a virtual endocrine organ, and it has been proved that the intestinal microbial balance and the steady-state becomes a requisite for animal maintenance organism health (Jiao *et al.* 2019). Generally, high microbial diversity was regarded to be associated with a healthy intestinal micro-ecosystem, while loss of diversity seems to be related to the disease (Pradhan *et al.* 2019). Early weaning is a common practice in modern sheep farming at present, but the immune and digestive system of early-weaned lambs were immature. The colonization of the suckling lamb intestinal microbiome during the first few months of life is a period of remarkable immaturity and fluctuation. The relatively indigestible solid

Table 2: Effects of BRYP on immunoglobulins and cytokines in intestinal mucosa (mg/g)

	The control group			The experimental group			
Item	Jejumum	Colon	Cecum	Jejumum	Colon	Cecum	*P*-value
IL-1 (pg/g)	3.86 ± 0.44	4.27 ± 0.83	4.01 ± 0.51	3.25 ± 0.74	3.88 ± 0.76	3.96 ± 0.25	0.739
IL-6 (pg/g)	3.05 ± 0.13a	3.96 ± 0.04b	3.71 ± 0.06b	2.98 ± 0.17a	3.63 ± 0.03b	3.15 ± 0.04a	0.038
IL-10 (pg/g)	2.05 ± 0.16a	3.41 ± 0.20b	3.54 ± 0.22b	2.78 ± 0.04ab	4.05 ± 0.39c	3.79 ± 0.31c	0.014
SIgA (mg/g)	10.28 ± 0.56a	13.69 ± 0.40b	14.27 ± 0.59b	11.44 ± 0.35a	14.52 ± 0.55b	16.03 ± 0.39c	0.036
IgG (mg/g)	23.94 ± 6.95	27.38 ± 4.67	29.55 ± 9.31	26.96 ± 7.35	28.87 ± 5.67	29.30 ± 6.52	0.855
TNF-α (pg/g)	4.84 ± 0.31b	4.82 ± 0.31b	4.81 ± 0.73b	4.80 ± 0.56b	4.81 ± 0.18b	4.43 ± 0.81a	0.048
IFN-γ (pg/g)	3.82 ± 0.02	4.05 ± 0.25	4.12 ± 0.20	3.59 ± 0.11	3.52 ± 0.42	4.05 ± 0.19	0.062

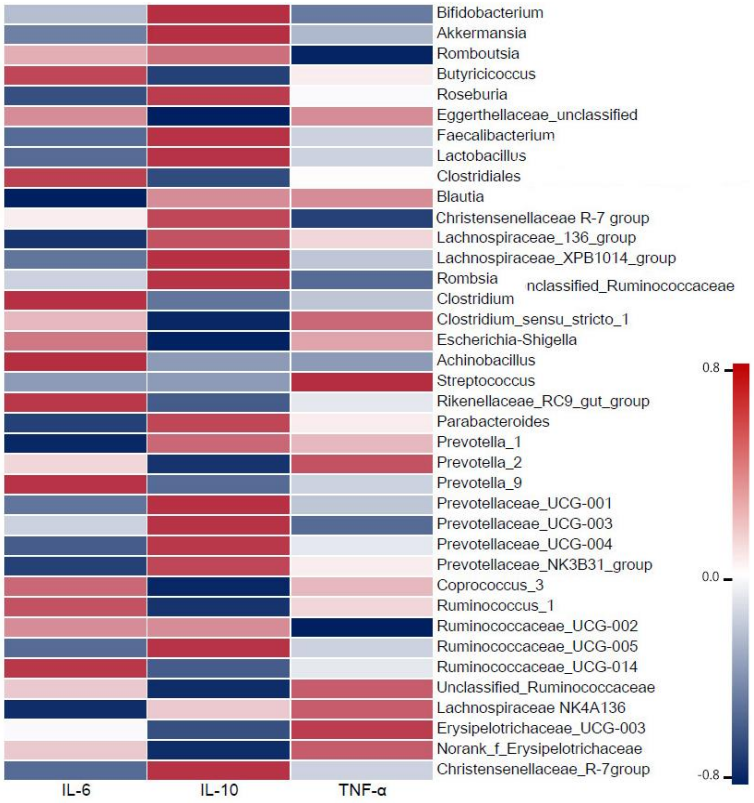

Fig. 9: Correlation Heatmap between BRYP-stimulated significantly changes in immune indices and intestinal bacteria in two groups

food will contribute to the risks of infection by pathogenic bacteria, acute diarrhea, and gastro-enteritis (Mao *et al.* 2019). In the early months, the density and diversity of intestinal microflora are constantly broadening in response to new environmental exposures until achieving a stable adult-like intestinal microbial composition (Blasco *et al.* 2019).

Most polysaccharides, such as yeast wall polysaccharides, have been proven to exert immunomodulatory, antiulcer, antioxidant, antitumor, and regulation of microbial composition (Mandal and Sahi 2018). BRYP is one of the main bioactive constituents in *Boulardii* yeast, which has been wildly used to treat diarrhea in babies. The mechanisms that *Boulardii* yeast wall polysaccharides could regulate intestinal microbial communities as follows: 1) BRYP is hard to be digested in the digestive tract, but BRYP could be used by intestinal probiotics, such as *Bifidobacterium* and *Lactobacillus*, which could promote the growth of prebiotics, but inhibit the growth of pernicious bacteria (Dong *et al.* 2019). 2) There was an interaction between intestinal bacteria and cytokines. Such interaction could be adjusted by BRYP-induced alteration of pathogen-associated molecular patterns (Singu *et al.* 2020). 3) The harmful and pathogenic bacteria could be absorbed in the large construction of BRYP (Méabed *et al.* 2019). It has been reported that the supplementation of *Pichia guilliermondii* cell polysaccharides significantly decreased the level of pH, and the number of pathogenic in the intestinal of chickens (Shanmugasundaram *et al.* 2014). The addition of yeast wall polysaccharides significantly increased the Alpha diversity of intestinal microbial in calf rumen (Jinjin *et al.* 2018). After 150 g/t yeast wall polysaccharides as supplementation were fed to 28 old-days piglets, the results showed that the

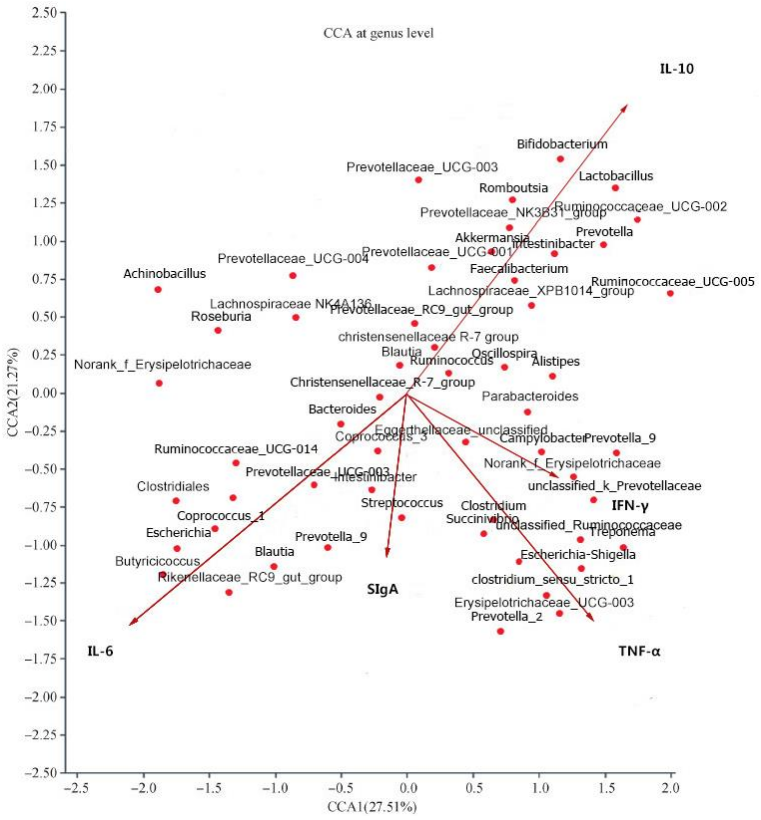

Fig. 10: CCA analysis results of intestinal microflora composition and immunologic factors

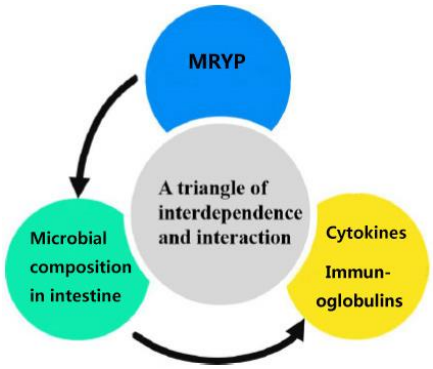

Fig. 11: A triangle of interdependence and interaction of MRYP, microflora composition and immune index

number of *Escherichia*, *Lactobacillus* were significantly increased in ileum and caecum (Murphy *et al.* 2013). After 250 g/t yeast wall polysaccharides as supplementation were fed to piglets, the results indicated that the number of *Escherichia* was significantly decreased in the caecum and colon (Sweeney *et al.* 2012).

Now-a-days, lots of technologies of high-throughput sequencing technologies, such as 16 Sr RNA, 26Sr RNA, 26Sr DNA, have been extensively used to analyze the composition of the intestinal microbial composition in human beings and animals. Besides, analysis of α-diversity involves a comparison of mean species richness, uniformity, and diversity found in two or more sets of samples using analysis of variance (Crist *et al.* 2003). In this study, the alpha-diversity was analyzed by using the ACE, Chao, Shannon, and Simpson diversity index. These results showed that the addition of 0.5% BRYP in milk replacer significantly increased the richness of intestinal microflora in the cecum and colon by analysis of Chao ($P < 0.05$). However, we found by analysis of Shannon and Simpson index that 0.5% BRYP in milk replacer significantly decreased the diversity of intestinal microflora in the colon ($P < 0.05$). These results indicated that 0.5% BRYP as a kind of supplement increased the species of intestinal microflora in the cecum and colon,

but decreased the uniformity of microflora species in the colon of early-weaned lamb. Besides, the increasing ACE data indicated that the abundance of microbial taxa increased after 0.5% BRYP as a supplement added into the basal milk replacer. Besides, it has been reported that fat deposits were related to the diversity of intestinal microflora, and the diversity of intestinal microflora of obesity was lower than a normal person with high probability (Aatsinki *et al.* 2018). In our feeding trail, we found that the average lambs' neck fat of 0.5% BRYP group was higher than the basal milk replacer group, which speculated that there might be a correlation ship between fat deposits and diversity of intestinal microflora in lambs.

For the first time, our study analyzed the addition of 0.5% BRYP in basal milk replacer altered the taxonomic composition of cecum and ileum microbial communities in early-weaned lambs. In this study, we found that *Firmicutes* and *Bacteroidetes* were two major phyla in the early-weaned lamb intestine. Besides, we found that the relative richness of *Bacteroidetes* was significantly increased by 0.5% BRYP, but the addition of 0.5% BRYP significantly decreased the relative richness of *Proteobacteria*. These results inferred that the risk of the infection by pathogenic in intestinal was decreased by BRYP, and increased the digestion and absorption of carbohydrate and fat in the digestive tract. Danzeisen has reported that the *Firmicutes* and *Bacteroidetes* were the predominant phyla within the caecum at all time-points (Danzeisen *et al.* 2011). Jumpertz reported that the increased *Firmicutes* and *Bacteroidete* in the intestine were related to the nutrient absorption (Jumpertz *et al.* 2011).

Besides, the growth of harmful microbes and pathogenic might multiply rapidly when the relative richness of *Proteobacteria* increased in the intestinal, and the increasing of harmful microbes and pathogenic might could result in dysbacteriosis, diarrhea, and gastro-enteritis in breeding production (Ramayo-Caldas *et al.* 2016). Those previous reports agreed to our results.

Generally, *Lactobacillus*, *Bacteroides*, *Prevotella*, *Oscillospira*, *Akkermansia,* and *Bifidobacterium* were probiotics in our intestine (Ley *et al.* 2006). However, most of *Escherichia* and *Streptococcus* were pernicious microbes (Isaacson and Kim 2012). *Lactobacillus* could produce bacteriocin-like substances, which were often active against related species of bacteria and eradicate neighboring bacteria by attaching themselves to receptors on their surfaces. Besides, Lactic acids, Acetic, and Biotin were produced by *Lactobacillus* could decrease intestinal pH to inhibit the growth of harmful bacteria (Chen *et al.* 2018b). *Prevotella* was another advantage microbe composition in the intestine. *Prevotella* could digest carbohydrates and protein, and it participated in Polysaccharide degradation, amino acid metabolism in rumen and intestinal (Fang *et al.* 2017). The *Oscillospira* could decrease in a person with the inflammatory response. Therefore, there is a negative correlation between the level of inflammatory response and the number of *Oscillospira* (Kovatcheva-Datchary *et al.* 2015). The abundance of *Akkermansia* was negatively correlated with levels of IL–6 and free fatty acids in serum. Besides, *Akkermansia* have the capacity to ameliorate inflammatory response, guard intestinal epithelial cells and strengthen mucosal barrier function (Ashrafian *et al.* 2019). *Bifidobacteria* could synthesize antimicrobial compounds such as bacteriocins, and other organic acids, which could control the growth and reproduction of the harmful microbes in the intestinal (Modrackova *et al.* 2020). On the other hand, *Escherichia* is a kind of common harmful bacteria in the intestinal, such as pathogenic *Escherichia coli* (EPEC), Enterotoxigenic E. *coli* (ETEC), *Enteroinvasive E. coli* (EIEC), and *Enterohaemorrhagic E. coli* (EHEC) can produce toxins, known as Vero toxins or Shiga toxins, which damage mucosa cells of the intestine and the kidneys (Sobhy *et al.* 2020). Besides, *Streptococcus* could produce toxins, such as streptolysin, pyrogenic exotoxin, hyaluronidase, streptodornase, streptokinase, and leipoteichoic acid (LTA), which can cause the imbalance of intestinal flora, bacterial translocation and decrease intestinal barrier function (Mabrouk *et al.* 2019). In this study, 0.5% BRYP as a supplement added into basal milk replacer significantly enhanced the relative richness of *Lactobacillus*, *Prevotella*, *Oscillospira,* and *Bifidobacteria*, but significantly decreased the *Escherichia* and *Streptococcus* in the lamb intestine, which indicated that addition of 0.5% BRYP could increase the proportion of probiotics, but decrease the proportion of harmful bacteria. In a word, BRYP improved the relative richness of beneficial bacteria, but suppressed the relative richness of harmful bacteria in the lamb intestine.

Some pieces of evidence have suggested that there are interactions between immune indices and intestinal bacteria (Ansaldo *et al.* 2019). Interleukin-6 (IL–6) as one of the sensitive indexes of early diagnosis for acute infection could increase and activate T lymphocyte and B lymphocyte (Huang *et al.* 2018). The immune response could be adjusted by IL-6, which acting an important role in anti-infection immunity, complement system, and excessive immune response (Ouyang and O'Garra 2019). Interleukin (IL)-10 is a kind of soluble protein, which exhibits a wide range of both immunostimulatory and immunosuppressive properties. The excessive immune response must often have followed in the intestine without or lack of IL–10 (Fiorentino *et al.* 2016). TNF-a is a pro-inflammatory cytokine known to have a crucial cell factor in the initial host response to infections and the pathogenesis of various chronic immune-mediated diseases (Reinke *et al.* 2020). These confirm the pivotal role and features underline of TNF-a in the immune system and in particular in the area of cell-mediated immune responses. However, massive TNF-a will occur serious excessive immune response in the intestine, and will lead to acute gastro-enteritis and acute diarrhea (Yang *et al.* 2019b). In this study, we found that there was a correlation between intestinal bacteria and immune index. For example,

Akkermansia had a positive correlation with IL–10, but had a negative correlation with IL–10 and TNF-α; In contrast, *Escherichia-Shigella* had a negative correlation with IL–10, but had a positive correlation with IL-6 and TNF-α.

Therefore, we can speculate there was a triangle of interdependence and interaction, which was composed of *Boulardii* yeast wall polysaccharide (BRYP), intestinal microbial composition, and immune index, as shown in Fig. 11. First of all, BRYP as a supplement altered the intestinal microbial composition. In this study, the relative abundance of *Bifidobacterium*, *Lactobacillus*, *Akkermansia* were increased, but the relative abundance of *Escherichia-Shigella*, *Clostridiales*, and *Streptococcus* were decreased. After that, the changed intestinal microbial composition enhanced the concentration of IL–10, but decreased the concentration of IL–6 and TNF-α. In a word, BRYP not only improved the intestinal microbial composition, but also indirectly suppressed the level of proinflammatory cytokines and increased the level of tolerance cytokine.

Conclusion

Compared with the control group, the richness of OTUs of the experimental group was significantly increased in the jejunum, cecum, and colon, respectively ($P < 0.05$). Compared with the control group, the species richness of the experimental group in the cecum and colon were significantly enhanced, but the diversity of species of the experimental group was decreased in the colon ($P < 0.05$). *Bacteroidetes* and *Firmicutes* were the most abundant phyla in all intestinal segments both in the control group and experimental groups, and the relative abundance of *Bacteroidetes* in the experimental group was significantly enhanced, but the *Proteobacteria* was significantly decreased in the jejunum, cecum, and colon ($P < 0.05$), compared with the control group. In the jejunum, the relative abundance of *Lactobacillus*, *Prevotella*, and *Fibrobacter* of the experimental group were significantly enhanced than that of the control group, but the *Ruminobacter* was significantly decreased ($P < 0.05$); In the cecum, the relative abundance of *Bacteroides*, *Lactobacillus*, *Oscillospira* and *Bifidobacterium* of the experimental group were significantly enhanced than that of the control group, but the *Blautia* were significantly decreased ($P < 0.05$); In the colon, the relative abundance of *Akkermansia*, *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium* of the experimental group were significantly enhanced than that of the control group, but the *Prevotella*, *Streptococcus*, and *Escherichia* were significantly decreased ($P < 0.05$). According to the results of Heat maps and Canonical correlation analysis (CCA), there were significant correlations between intestinal immune indices (IL–6, IL–10, TNF-α) and intestinal microbial composition in the colon ($P < 0.05$). These findings suggested that BRYP may contribute to the promotion of the proportion of helpful microbial populations and enhancing the balance of intestinal microflora. Besides, the changed intestinal microflora composition may indirectly induce mucosal immune interactions, which may improve local immune function, but suppress the inflammatory response of the bottom of intestinal mucosa in early-weaned lambs.

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Author Contributions

Mengjian Liu: Conceptualization (equal); Methodology (supporting); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing-original draft (equal); Writing-review & editing (equal); **Wenju Zhang:** Supervision (equal); Formal analysis (supporting); Validation (equal); Resources (supporting); Project administration (equal); **Jun Yao:** Software (supporting); **Junli Niu:** Data curation (supporting); Formal analysis (supporting); Software (equal); Visualization (equal).

Conflicts of Interest

No conflict of interest exists in the submission of this manuscript, and the manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed

Data Availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study

Ethical Approval

This study's protocols and procedures were ethically reviewed and approved by the Animal Welfare Committee of Shihezi University with the ethical code: A2019-156-01

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