Effects Of Three Kinds of Complex Probiotics on Intestinal Flora and Intestinal Tissue Morphology in Diarrhea Mice with Intestinal Flora Imbalance

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Abstract: To study the effects of three kinds of complex probiotics (No.1, No.2 and No.3) on the body weight, intestinal flora and intestinal mucosal function of dysbacteriosis model mice. In this experiment, the model mice with dysbacteriosis caused by antibiotics were taken as the research object. After successful modeling, they were randomly divided into three groups: test group I (gavage of No.1 bacteria), test group II (gavage of No.3 bacteria), test group III (gavage of No.3 bacteria), and normal group and model group without any probiotics. After the middle period of the experiment, the body weight and daily gain of mice during the experimental period were measured Three mice in each group were treated to make tissue sections for morphological observation. The intestinal flora was cultured and counted. The results showed that compared with the model group, continuous supplementation of No. 1 and No. 2 compound probiotics could effectively promote the growth of mice in the state of flora imbalance; Promote the colonization and growth of beneficial bacteria in each intestinal segment, promote the growth of intestinal villi in mice, enhance the absorption of nutrients in the intestine, and increase the ratio of the length of intestinal villi to the depth of glandular fossa. The above results indicated that supplementation of probiotics I and II could obviously relieve stress, promote growth, increase the number of beneficial bacteria in intestinal tract and repair mucosal barrier in mice with dysbacteriosis, which provided theoretical basis for its application in promoting growth and improving intestinal microecosystem of livestock and poultry.

1. Introduction

There is a close relationship between human host and intestinal microflora. Intestinal microflora are antagonistic or promotive microorganisms, which protect the intestinal tract from colonization by exogenous pathogens. In addition, intestinal microflora plays a key role in providing nutrition and regulating host immune homeostasis. Studies on probiotics in recent years have shown that feeding probiotics can help animals restore the integrity and function of intestinal barrier, increase intestinal mucus, promote nutritional digestion, improve the growth performance of livestock and poultry, improve intestinal flora, inhibit the growth of harmful bacteria, reduce diarrhea, prevent diseases, improve the feeding environment and improve the quality of livestock and poultry meat [1-3]. Therefore, the intervention and management of intestinal micro-ecosystem with probiotics has attracted much attention in relieving intestinal stress, preventing diseases, correcting AAD, stimulating growth potential and improving growth performance. According to the principle of "aerobic bacteria, facultative aerobic bacteria and anaerobic bacteria", three kinds of compound probiotics were compounded. Comparative study on the effects of three kinds of complex probiotics on compensatory growth, intestinal flora and intestinal mucosal barrier function of dysbacteriosis model mice provided theoretical basis for the application of probiotics in promoting growth and improving intestinal microecosystem of livestock and poultry.

2. Materials and Methods

2.1 Laboratory animals, strains and main reagents

70 SPF Kunming mice, half male and half female, 25 days old, weighing 17 g ~21 g, were purchased from Liaoning Changsheng Biological Co., Ltd.; Lactobacillus plantarum CGMCC12436, Enterococcus faecium R-026, Streptococcus faecalis T-110, donated by Beijing Feed Research Institute; Bacillus mesentericus TO-A, Bacillus coagulans DH156, Saccharomyces cerevisiae BLNJ03, CCTCCNO: M2015209, Clostetidium butyricum QA-08, the strains are reserved by the Animal Science Department of Shenyang Institute of Technology. Gentamicin sulfate was purchased from Henan Runhong Pharmaceutical Co., Ltd. (batch number: 1707111); Cefradine was purchased from Guangzhou Baiyunshan Pharmaceutical Group Co., Ltd. (batch number: 2160032); The mixture of gentamicin sulfate and cefradine was prepared into 62.5 g/L antibiotic solution, which was stored in refrigerator at 4℃. Nutrient broth, YEPD liquid medium, MRS broth, brain heart extract broth, sodium azide aescine crystal violet agar medium, XLD agar medium, Sabouraud agar medium, BS agar medium, LBS agar medium, etc. are purchased from Qingdao Haibo Biotechnology Co., Ltd.

2.2 Preparation of compound probiotics

Purified strains such as aerobic bacteria (Bacillus coagulans DH156, Bacillus mesenteroides TO-A), facultative anaerobic bacteria (Enterococcus faecalis T-110, Enterococcus faecalis R-026, Candida utilis BLNJ03, Lactobacillus plantarum CGMCC12436) and anaerobic bacteria (Clostridium butyricum QA-08) were rejuvenated, and then expanded and cultured in liquid medium. When in use, collect bacteria by centrifugation, and adjust the number of bacteria according to the formula with normal saline, namely: Compound probiotics No.1(Bacillus coagulans DH156 0.4×10^8 cfu/mL, Bacillus mesenteroides TO-A 0.5×10^8 cfu/mL, Clostridium butyricum QA-08 4.6×10^8 cfu/mL, Enterococcus faecium R-026 1×10^8 cfu /mL, Enterococcus faecalis T-110 1×10^8 cfu/mL); Compound probiotics No.2(Lactobacillus plantarum CGMCC12436 1×10^8 cfu/mL, Clostridium butyricum QA-08 4.6×10^8 cfu/mL, Bacillus mesenteroides TO-A 0.5×10^8 cfu/mL, Enterococcus faecalis T-110 1×10^8 cfu /mL, Bacillus coagulans DH156 0.4×10^8 cfu/mL); Compound probiotics No.3 (BLNJ03 Candida utilis 3×10^8 cfu/mL, Clostridium butyricum QA-08 4.6×10^8 cfu/mL, Bacillus mesenteroides TO-A 0.5×10^8 cfu/mL, Lactobacillus plantarum CGMCC12436 1×10^8 cfu /mL, Bacillus coagulans DH156 0.4×10^8 cfu/mL).

2.3 Grouping, handling and feeding management of experimental animals

Sixty SPF Kunming mice were randomly divided into five groups, with 12 mice in each group, namely normal group, model group, experimental group I, experimental group II and experimental group III. They were raised in cages and adaptively for 3 days. Before the formal test, the mice were made a model of diarrhea with dysbacteriosis in advance, and the modeling method was carried out with reference to the literature [4-5], that is, the normal group was given 0.35 mL/, and the other groups were given 0.35 ml/; Twice a day for 7 days, the model was successfully established when feces became thin and soft, and mice were depressed. See table 1.1 for the dosage and method of intragastric administration of mice in each group during the formal trial period of 18 days. Feeding conditions are controlled at 22℃~24℃, natural lighting, free drinking and feeding.

Table 1.1 Animal grouping and treatment

Group	To deal with	Dose mL one/each time	Duration (d)
Normal group	NS.p.o., q.12 h.	1	18
Model group	NS.p.o., q.12 h.	1	18
Test groupI	No.1 bacteria, p.o., q.12 h.	1	18
Test group II	Bacteria No.2, p.o., q.12 h.	1	18
Test group III	Bacteria No.3, p.o., q.12 h.	1	18

Note: NS: normal saline; No.1 bacteria: complex probiotics I; No.2 bacteria: complex probiotics II; No.3 bacteria: complex probiotics III; P.O.: one/each time; q.12.h: Every 12 hours.

2.4 Average weight gain measurement

The weight of mice in each group was measured every three days during the experiment, and the weight difference and weight change rate were calculated.

Weight change rate = (final weight-weight after modeling)/weight after modeling ×100%

2.5 Determination of intestinal flora in mice

After the mice were killed by decapitation, the abdominal cavity was opened aseptically, and the intestinal contents of jejunum, ileum, cecum and colon were quickly weighed, which were mixed with sterilized normal saline at a ratio of 1:9 for later use. In addition, several sterilized test tubes were taken for multiple dilution, with a total of 7 gradients of $10^1 \sim 10^7$. Different intestinal segments and culture objects have different gradients. For example, in jejunum and ileum, enterococcus and Escherichia coli are $10^2 \sim 10^4$; 10^3-10^5 yeasts; Bifidobacterium and lactic acid bacteria are $10^4 \sim 10^6$; In cecum and colon, enterococcus and yeast are $10^3 \sim 10^5$; Escherichia coli, Bifidobacterium and lactic acid bacteria are $10^4 \sim 10^6$. Take 10 microliters of diluted samples with a microsampler, and drop the seeds in each selective plate culture medium which has been zoned. One plate drops seeds in three gradients. After sample collection, dilution and seed dropping, culture was carried out on the same day. The cultivation methods are aerobic and anaerobic according to the different objects (see Table 1.2). After aerobic or anaerobic culture, count, and finally calculate the number of bacteria contained in each gram of sample, which is expressed as the logarithm of the number of bacteria in each gram of intestinal contents (Log_{10}(cfu/g)).

Table 1.2 Microbial culture conditions of each intestinal segment

Projects	Culture conditions	Incubation time
Enterococcus	Aerobic;37℃	24 h
Escherichia coli	Aerobic;37℃	24 h
Yeast	Aerobic;37℃	72 h
Bifidobacterium	Anaerobic;37℃	48 h
Lactic acid bacteria	Anaerobic;37℃	48 h

2.6 Analysis of intestinal tissue morphology in mice

After the mice were killed by taking off their necks, the abdominal cavity was opened, and about 1-2 cm intestinal rings were cut out at proper positions of duodenum, jejunum, ileum, cecum and colon. Rinse it with sterilized cold physiological saline, and make paraffin section. After staining and sealing, the samples were observed under an optical microscope, and the images were collected and measured by Image-Pro Plus6.0. About 5 ~ 10 villi were selected from each section to measure the villi length, depth of glandular fossa and thickness of intestinal wall.

2.7 Data analysis

The experimental data were expressed by mean standard deviation ($\bar{x} \pm$SD), and one-way variance analysis was performed by SPSS24.0 statistical software. Tukey method was used for comparison between groups, and nonparametric test was used for analysis of data with uneven variance, with $p<0.05$ as significant difference.

3. Result

3.1 Effect of compound probiotics on body weight of diarrhea mice with dysbacteriosis

After 7 days of antibiotic modeling, compared with the normal group, the model mice showed AAD symptoms such as listlessness, decreased food intake, reddish perianal and increased fecal water content, indicating that the model mice with dysbacteriosis were successfully modeled. After 18 days of continuous oral administration of probiotics, the weight changes of mice in each group were recorded. The results showed that there was no significant difference between the weight changes and

the rate of change of mice in each experimental group ($p>0.05$), but compared with the model group, the average weight gain of mice in experimental groups I, II and III was significantly increased (see Table 2.1). The results showed that continuous supplementation of three kinds of complex probiotics can effectively relieve the growth slowdown of model mice in AAD state, help correct intestinal flora, enhance the digestive function of mice and help restore their physique.

Table 2.1 Changes of body weight of mice in each experimental group

Group	Weight change (g)	Weight change rate (%)
Normal group	7.25±5.22	28.78±26.97
Model group	4.78±2.06	16.45±7.04
Group I	6.05±7.13	18.45±3.53
Group II	7.13±1.82	22.02±6.19
Group III	6.50±1.06	22.96±3.67

3.2 Effect of compound probiotics on the number of intestinal florae in diarrhea mice with dysbacteriosis

After the experimental period, the number of intestinal florae in mice was measured. It can be seen from fig. 1 that in jejunum, ileum, cecum and colon, the amount of flora measured in each experimental group is higher than that in model group, and the amount of flora measured in some intestinal segments is higher than or equal to that in normal group. In jejunum, the numbers of enterococci, yeasts and lactic acid bacteria in test group I and II were significantly different from those in model group ($P < 0.01$), and the numbers of Escherichia coli in test group I and Bifidobacterium in test group III were also significantly different from those in model group ($P < 0.01$). In ileum, the number of Escherichia coli, yeast in experimental group I was significantly different from that in model group ($P < 0.01$), the number of Escherichia coli, Bifidobacterium and lactic acid bacteria in experimental group II was significantly different from that in model group ($P < 0.01$). In cecum, the number of enterococci in each test group was significantly different from that in model group ($P < 0.01$), and the number of Escherichia coli and lactic acid bacteria in test group I, yeast in test group II and Bifidobacterium in test group III were significantly different from that in model group ($P < 0.01$). In colon, the number of Escherichia coli and yeast in test group I was significantly different from that in model group ($P < 0.01$), the number of enterococci and lactic acid bacteria in test groups II and III was significantly different from that in model group ($P < 0.01$). In addition, the number of other florae measured in each experiment is as shown in the figure. Although there is no significant difference with the model group, the values of each group are higher than those of the model group, indicating that continuous supplementation of three complex probiotics can increase the number of beneficial bacteria in colon (see Figure 2.1)

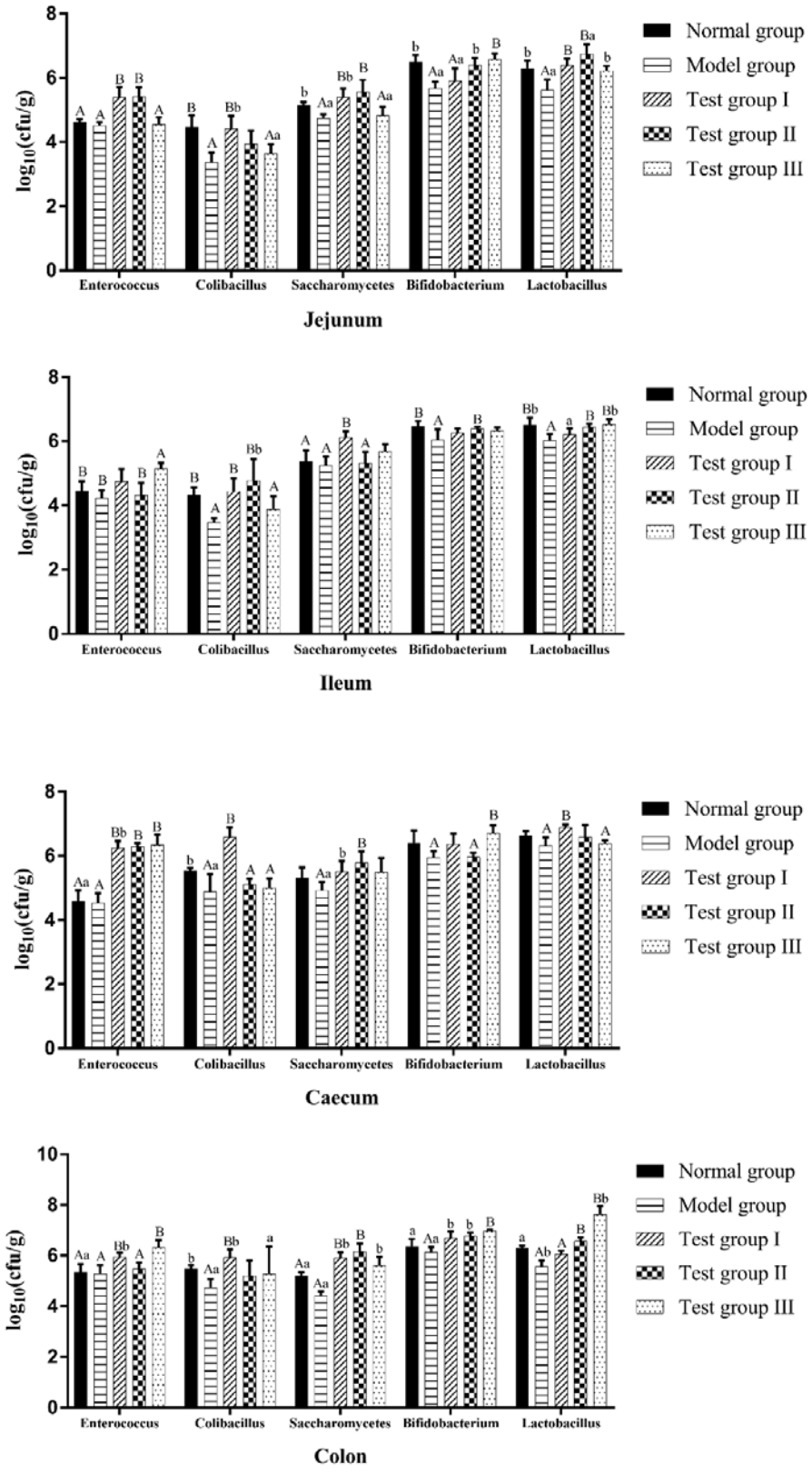

Fig.1 Changes of intestinal microflora in mice of each test group

Note: Different lowercase letters indicate significant differences ($P < 0.05$), while different uppercase letters indicate extremely significant differences ($P < 0.01$). The following table is the same.

3.3 Effect of compound probiotics on intestinal tissue morphology of diarrhea mice with dysbacteriosis

After the end of the experimental period, the mice in each experimental group were treated, and the intestinal tissue morphology was observed and determined. In jejunum, the villi length of model group was significantly lower than that of normal group ($P < 0.01$) and significantly lower than that of experimental group I ($P < 0.05$). However, there was no significant difference in the depth of glandular fossa among the experimental groups ($P > 0.05$). The cashmere ratio of the model group was significantly lower than that of the experimental group I ($P < 0.01$), while the cashmere ratio of the model group was significantly higher than that of the normal group ($P < 0.05$). In ileum, the ratio of villous glands in model group was significantly lower than that in normal group ($P < 0.01$) and significantly lower than that in experimental group I ($P < 0.05$). However, there was no difference in villus length, crypt depth and intestinal wall thickness in ileum ($P > 0.05$). In duodenum, the villus length of model group was significantly lower than that of normal group ($P < 0.01$) and significantly lower than that of experimental group I ($P < 0.05$). Compared with the normal group, the cashmere ratio of the model group decreased significantly ($P < 0.01$), and that of the experimental group I decreased significantly ($P < 0.05$). In duodenum, the villus length of the model group was significantly lower than that of the normal group ($P < 0.01$), and there was no difference with other experimental groups ($P > 0.05$), but it was significantly lower. Compared with the normal group, the depth of glandular fossa in the model group was significantly higher ($P < 0.05$), and there was no difference with other experimental groups, but there was an obvious increasing trend. There was no significant difference in intestinal wall thickness among the experimental groups ($P > 0.05$), and the villous gland ratio of the model group was significantly lower than that of the normal group ($P < 0.01$), but it was significantly lower than that of other experimental groups. The results showed that continuous supplementation of three kinds of complex probiotics could alleviate ADD symptoms, increase villus length, reduce the depth of glandular fossa, enhance absorption function, repair intestinal tissue and promote its absorption of nutrients (see Table 2.2).

Table 2.2 Changes of intestinal tissue morphology in mice of each test group

Items（mm）	C group	Model group	Test group I	Test group II	Test group III
Jejunum					
Villus height	0.50 ± 0.05^{B}	0.21 ± 0.06^{Aa}	0.48 ± 0.16^{b}	0.37 ± 0.11	0.25 ± 0.02
Crypt depth	0.11 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	0.09 ± 0.02
Intestinal wall thickness	0.05 ± 0.01	0.06 ± 0.02	0.04 ± 0.01^{A}	0.05 ± 0.01	0.09 ± 0.01^{B}
Cashmere gland ratio V/C	4.78 ± 1.13^{b}	2.05 ± 0.51^{Ba}	5.37 ± 0.87^{A}	3.82 ± 0.82	2.90 ± 0.44
Ileum					
Villus height	0.17 ± 0.09	0.11 ± 0.02	0.19 ± 0.03	0.15 ± 0.09	0.12 ± 0.02
Crypt depth	0.08 ± 0.04	0.08 ± 0.01	0.06 ± 0.02	0.08 ± 0.05	0.08 ± 0.01
Intestinal wall thickness	0.06 ± 0.03	0.06 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.02
Cashmere gland ratio V/C	2.20 ± 0.09^{Bb}	1.38 ± 0.09^{Aa}	1.99 ± 0.05^{b}	1.77 ± 0.12	1.53 ± 0.06^{a}
Duodenum					
Villus height	0.59 ± 0.11^{B}	0.26 ± 0.10^{Aa}	0.51 ± 0.08^{b}	0.38 ± 0.09	0.39 ± 0.07
Crypt depth	0.08 ± 0.01^{a}	0.14 ± 0.04^{b}	0.11 ± 0.01	0.10 ± 0.02	0.13 ± 0.02^{b}
Intestinal wall thickness	0.04 ± 0.01	0.10 ± 0.06	0.05 ± 0.01	0.07 ± 0.03	0.06 ± 0.01
Cashmere gland ratio V/C	7.17 ± 1.75^{Bb}	1.87 ± 0.51^{Aa}	4.80 ± 0.39^{b}	3.97 ± 0.34	3.07 ± 0.21^{a}

Note: Different lowercase letters indicate significant differences ($p<0.05$), and different uppercase letters indicate extremely significant differences ($p<0.01$).

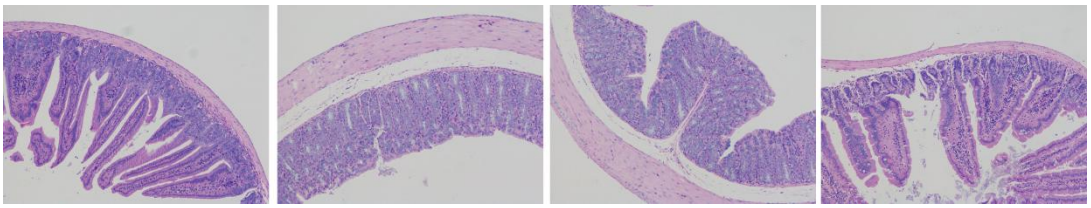

Fig 2 Morphology of intestinal mucosa in normal mice (from left to right: jejunum, ileum, colon, duodenum)

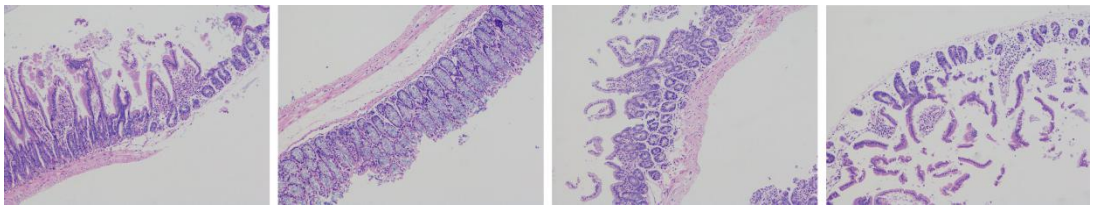

Fig 3 Intestinal mucosa morphology of mice in model group (from left to right: jejunum, ileum, colon, duodenum)

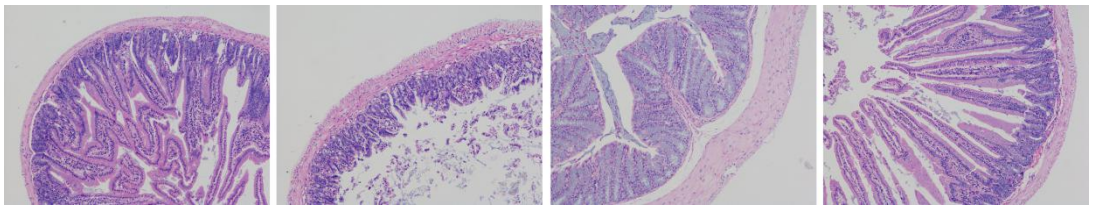

Fig 4 Intestinal mucosal tissue morphology in experimental group (from left to right: jejunum, ileum, colon, duodenum)

4. Discussion

Under normal circumstances, there is a complex balance between intestinal flora in the gastrointestinal tract, which helps the body transform and absorb nutrients. Many factors can cause dysbacteriosis and lead to diseases. At present, research shows that antibiotic modeling can cause typical tertiary flora imbalance on intestinal mucosa, which leads to the decrease of intestinal flora diversity and abnormal proliferation of some pathogenic bacteria [6, 7], causing AAD symptoms. In this experiment, feeding antibiotics for 7 consecutive days can cause the model mice to show AAD symptoms of intestinal flora disorder, such as listlessness, decreased food intake, reddish perianal region and increased fecal water content. A large number of studies have confirmed that adding probiotics to feed can significantly improve the growth performance of animals [8-10]. The results of this experiment show that continuous supplementation of three kinds of compound probiotics can promote the growth of mice in dysbacteriosis with ADD symptoms. Compared with the model group, the weight changes of test group I, test group II and test group III are obviously higher, but there is no obvious difference among the test groups.

The length of villi and the depth of crypt are indicators to measure the growth and development of intestinal epithelial cells. Intestinal villi are the protrusions of the epithelium and lamina propria of intestinal mucosa extending to the intestinal cavity, with different shapes, especially the duodenum and the head of jejunum. The formation of villi can enlarge the absorption area of small intestine. The height of intestinal villi increases and the absorption area of intestine increases [11]. The epithelium of villi root sinks to the lamina propria to form intestinal glandular fossa, also known as intestinal crypt, which opens directly into the intestinal cavity. Under normal circumstances, the cells at the base of crypt constantly differentiate and migrate to the end of villi, forming intestinal epithelial cells with absorptive capacity to supplement the intestinal epithelium with normal shedding of villi. The depth of the glandular fossa reflects the cell production rate, while the shallowing of the glandular fossa indicates that the cell maturation rate increases, the absorption function is enhanced [12], and

the intestinal mucosa can be quickly repaired. The ratio of villus length to glandular fossa depth comprehensively reflects the absorption energy of small intestine. The larger the ratio in a reasonable range, the stronger the absorption capacity of small intestine. The results of this study showed that three kinds of compound probiotics could increase the villus length and the ratio of villi to glands ($P < 0.01$ or $P < 0.05$) and reduce the depth of glandular fossa ($P < 0.01$ or $P < 0.05$), which indicated that the three kinds of compound probiotics used in the experiment could effectively repair and improve intestinal mucosa and promote the absorption of nutrients. More than 95% of normal intestinal flora are obligate anaerobic bacteria, while the proportion of facultative anaerobic and aerobic bacteria is very small, about 1%. According to the literature [13,14], the intestinal flora of diarrhea dogs is obviously out of balance, in which enterococcus, Bifidobacterium and lactic acid bacteria are significantly reduced, and the number of Escherichia coli is increased. Literature [15] showed that the number of bifidobacteria and lactobacilli in the intestinal tract of early weaned diarrhea piglets was significantly lower than that of healthy piglets, while the number of Escherichia coli and Clostridium increased significantly ($P < 0.05$), and there was no significant difference between other bacteria and healthy piglets ($P > 0.05$). The results showed that enterococci, yeasts, bifidobacteria and lactic acid bacteria in each intestinal segment of mice were significantly higher than those in model group ($P < 0.01$ or $P < 0.05$). Compared with model group, the number of E. coli in other intestinal segments was significantly higher or increased ($P < 0.01$ or $P < 0.05$). Mingqing He [16] found that aerobic Bacillus, Escherichia coli and Salmonella are normal flora in piglet intestinal tract. Gastrointestinal flora in normal state is beneficial to the host. With the progress of the experiment, three kinds of compound probiotics were continuously supplemented in the experimental group I, the experimental group II and the experimental group III, and the intestinal flora was restored, which may establish a new balance.

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