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Early Intervention Using Fecal Microbiota Transplantation Combined with Probiotics Influence the Growth Performance, Diarrhea, and Intestinal Barrier Function of Piglets

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Featured Application: The early intervention with fecal microbiota combining *Clostridium butyricum* and *Saccharomyces boulardii* (FMT+C+S) could improve the growth performance and reduce the diarrhea rate by improving intestinal barrier function and production of short-chain fatty acids in piglets. This study provides a new theoretical support for regulating gut microbiota and immune function in piglets and even in human neonates.

Abstract: Early intervention with fecal microbiota transplantation (FMT) improves the growth performance and intestinal barrier function of piglets. Accelerating intestinal oxygen concentration is beneficial for symbiotic bacterial colonization. *Saccharomyces boulardii* (SB) is an aerobic fungus, which may contribute to the colonization of anaerobic symbiotic bacteria by competing for oxygen. *Clostridium butyricum* (CB) improves intestinal barrier function and performance, via regulating the gut microbiota composition of piglets. The objective of this study was to investigate the effect of early intervention with FMT combining CB and SB on growth performance, diarrhea, and intestinal barrier function in piglets. A total of 77 litters of neonatal piglets assigned to one of six treatments, which treated with antibiotics (AB), placebo (CON), and FMT (FMT), FMT-added CB (FMT+C), FMT-added SB (FMT+S), and FMT-added CB and SB (FMT+C+S), respectively. FMT+C+S treated piglets had higher body weight (BW) and average daily gain (ADG) both in weaning and finial period, and it significantly increased the levels of fecal mucin-2 (MUC2), fecal short-chain fatty acids (SCFAs), and relative abundance of fecal *Lactobacillus* spp., and *Bifidobacterium* genus. Moreover, early intervention with FMT+C+S reduced the diarrhea rate during the experiment. FMT+C+S also decreased the level of plasma diamine oxidase (DAO) and D-lactate (D-LA), and relative abundance of fecal *E. coli* during the suckling period. In summary, early intervention with FMT combining CB and SB improved the growth performance, intestinal barrier function, fecal SCFAs concentration, and fecal *Lactobacillus* and *Bifidobacterium* of piglets.

Keywords: early intervention; growth performance; intestinal barrier function; diarrhea; piglets

1. Introduction

Diarrhea is a common and important disease which seriously affects the growth development and mortality of piglets, and restricts economic benefit of pig industry. Previous studies indicated that diarrhea of piglets is mainly related to the gut microbiota dysbiosis and intestinal barrier function [1–3]. Therefore, to promote development of intestinal barrier function is an important way to alleviate diarrhea and improve the growth performance of newborn piglets. Growing evidence has suggested that the gut microbiota plays an important role in the development of the intestinal barrier function [4–6]. Therefore, accelerating the colonization of gut microbiota of newborn piglets may be an effective method to promote maturity of intestinal barrier function, reduce diarrhea and improve the growth performance of piglets.

Gut microbiota has many roles benefiting the host. Many studies revealed that the early postnatal period is an early intervening window for regulating colonization and development of gut microbiota [7–9]. Fecal microbiota transplantation (FMT) is gradually being used in improving growth and regulating the development of gut microbiota in newborn piglets. Recent studies supported that feeding crossbred newborn piglets with FMT suspension of healthy adult pigs significantly promoted the growth performance and intestinal barrier function of newborn piglets [2,7,10]. Research indicated that the colonization of gut microbiota follows patterns of "first come, first served", homologous bacteria are more likely to colonize the recipient's gut, and hypoxic intestinal environment is more conducive to the colonization of symbiotic bacteria [11–13]. Probiotics exhibit a profound influence on maintaining intestinal homeostasis, regulating gut microbiota and reducing diarrhea [11,13]. *Saccharomyces boulardii* (SB) is beneficial for gut health [14–16] and could reduce the abundance of intestinal aerobic bacteria [17,18]. *Clostridium butyricum* (CB) is known as a regulator for gut health, and the dietary supplementation of CB can improve early intestinal barrier function [19–22]. However, whether SB or/and CB could improve the effect of early intervention remains elusive.

Here, we hypothesized that SB and CB may improve the effect of FMT through oxygen competition and enhancing the intestinal barrier function. Therefore, the objective of this study was to investigate the effects of FMT combining CB and SB in early intervention on growth performance, diarrhea rate, intestinal barrier function, fecal microbiota and production of short-chain fatty acids (SCFAs) in piglets, under an antibiotic-free condition; in addition, we set up the antibiotic-treated group as a positive control group.

2. Materials and Methods

The protocol for the animal experimental procedures was approved by Institutional Animal Care and Use Committee of Huazhong Agricultural University (Wuhan, China). The ethical number of this study is HZAUSW-2018-013.

2.1. Preparation of Fecal Microbiota Suspension of Donor Pigs

Six sows (Landrace × Large White) (Table S1), at 70 days of gestation, without any antibiotics treatment for more than 3 months, were used in this experiment as fecal donors. The fecal suspension was prepared as described by Pang et al. [23] and Cheng et al. [7]. We divided all the prepared fecal suspension into 4 parts on average: with no probiotics added for the FMT group, with 1.0×10^9 CFU/mL *C. butyricum* added for the FMT+C group, with 1.0×10^9 CFU/mL *S. boulardii* added for the FMT+S group, and with 1.0×10^9 CFU/mL *C. butyricum* and 1.0×10^9 CFU/mL *S. boulardii* added together for the FMT+C+S group. All the suspension was stored in liquid nitrogen.

2.2. Animals and Experimental Design

A total of 77 Landrace × Large White sows with 109 days of pregnancy and average parity of 3.77 ± 1.32 were randomly assigned in 6 groups: 13 in the antibiotic group (AB), 10 in the control group (CON), 15 in the fecal microbial transplantation group (FMT), 15 in the FMT-added *C. butyricum* group

(FMT+C), 15 in the FMT-added *S. boulardii* group (FMT+S), and 12 in the FMT-added *C. butyricum* and *S. boulardii* group (FMT+C+S) (Table S2). Sows were moved from the gestation pans to the farrowing rooms on day 109 of gestation. The sows and piglets were individually housed in farrowing pens with crates, slatted floors and heat pads for piglets. The diet of sows contained no probiotics and antibiotics. The sows were individually fed and had ad libitum access to water, and all the sows provided the same amount of feed per day during the experimental period. At parturition, each newborn piglet was weighed individually and the number of live-born piglets recorded.

2.3. Transplantation of Fecal Microbiota Experiment and Animal Management

Piglets in the AB group were accepted intramuscular injection of antibiotics (amoxicillin) for health care on age of 3d, and feed diet with antibiotic growth promoters at the age of 9 d. The piglets of other groups were treated after born as follows: oral inoculation placebo (CON), fecal suspension (FMT), fecal suspension with 1.0×10^9 CFU/mL *C. butyricum* (FMT+C), fecal suspension with 1.0×10^9 CFU/mL *S. boulardii* (FMT+S), and fecal suspension with 1.0×10^9 CFU/mL *C. butyricum* and 1.0×10^9 CFU/mL *S. boulardii* (FMT+C+S), respectively. The dosage of inoculant was 2.0 mL/piglet once daily in the first 3 days. There was exposure to antibiotic-free diets at the age of 9 d.

All piglets were weighed within 24 h after birth, and numbered each individual piglet with an ear-mark, then weighed once a week. The number of piglets per litter were adjusted to 11 to 13 (Table S2), and the excess piglets (such as weak ones) were fostered by unselected sow. All the piglets had ad libitum access to water and diet was available at the 9th day of age. Piglets were breast-fed by sows and weaned at the age of 28 days. After weaning, a total of 500 healthy piglets, with body weight more than 5.5 kg, were selected and fed for 5 weeks. Except for the AB piglets, no antibiotics ration and water were provided ad libitum for piglets.

2.4. Determinations of Growth Performance and Diarrhea Rate

Piglets were individually weighted weekly to determine average daily gain (ADG). The diarrhea rate was recorded every day during experimental period, and the occurrence of diarrhea was visually assessed and evaluated by individual scoring the consistency of the fecal matter between 15:00 to 18:00 each day. In brief, scores were 0, firm fecal, normal; 1, pasty fecal, slight diarrhea; 2, semi-liquid fecal, moderate diarrhea; or 3, liquid and unformed fecal, severe diarrhea [7,24]. The diarrhea rate was calculated as follows: Diarrhea rate (%) = (number of diarrhea piglets)/(Total number of experimental piglets × Experimental time (day)) × 100%. Diarrhea index = Total fecal scores/Total number of experimental piglets.

2.5. Sample Collections

At days 7, 14, 21, and 27 of the experiment during the suckling period (days of 127), one piglet of each litter was randomly selected, and at days of 35, 42, 49, and 56 day during the post-weaning period (days 28–63), 3 piglets of each replicate (n = 4 per treatment) were selected for blood samples via the anterior vena cava puncture (tubes containing heparin sodium) after an overnight fast, and this was centrifuged at 3000 rpm for 10 min. The same number of fresh fecal samples of piglets at the same time were collected. Samples were frozen at −80 °C.

2.6. Determination of Intestinal Barrier Biomarkers

The plasma levels of diamine oxidase (DAO), D-lactate (D-LA) and citrulline were measured to evaluate the gut barrier function of piglets. The level of DAO and D-LA were measured using an enzyme-linked immunosorbent assay (ELISA) test kit (mlbio, Shanghai, China) according to the manufacturer's instructions. High-performance liquid chromatography (HPLC) analysis was used to determine plasma citrulline levels.

2.7. Determinations of Fecal Short-Chain Fatty Acids and Fecal Succinate

Fecal succinate concentration was measured using an ELISA test kit (mlbio, Shanghai, China) according to the manufacturer's instructions. The SCFAs concentrations of fecal and plasma in day 14 and 21 were analyzed by a gas chromatographic method according to our previous study [7]. Total SCFAs in fecal were determined as the sum of analyzed acetate, propionate, butyrate, valerate, isobutyrate and isovalerate. All procedures were performed in duplicate.

2.8. DNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (PCR)

Total microbial DNA was extracted and purified from fecal samples on days 3, 7, 14, and 21 using a QIAamp DNA stool kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of DNA was assessed using a NanoDrop® ND-1000 Spectrophotometer. Real-time quantitative polymerase chain reaction (PCR) analyses were performed (CFX ConnectTM Real-time PCR Detection System; Bio-Rad) in a final reaction volume of 10 µL containing 4.4 µL of template DNA (50 ng/µL), 5 µL iTaq™ Universal SYBR Green Supermix (Bio-Rad) and 0.3 µL of each of forward and reverse primers. Thermal cycling conditions involved an initial denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and 65 °C for 1 min (Wei et al., 2015). Dissociation analyses of the PCR product were performed to confirm the specificity of the resulting PCR products. The primers used for the real-time PCR detection of selected genes are listed in Table 1.

Table 1. Primers used for absolute quantitative real-time polymerase chain reaction (PCR) in this study.

Target Group	Sequence of Primers (5′ to 3′)	Size (bp)	Annealing Temperature (°C)	Reference
Total bacteria	Forward: ACTCCTACGGGAGGCAGCAG Reverse: ATTACCGCGGCTGCTGG	175	60	Cheng et al., 2018
Lactobacillus spp.	Forward: CACCGCTACACATGGAG Reverse: TGGAAGATTCCCTACTGCT	341	58	Cheng et al., 2018
Escherichia coli	Forward: CATGCCGCGTGTATGAAGAA Reverse: TTTGCTCATTGACGTTACCCG Reverse: AATTCCGCCTACCTCTGCACT	96	60	Huijsdens et al., 2002
Bifidobacterium genus	Forward: TCGCGTC(C/T)GGTGTGAAAG Reverse: CCACATCCAGC(A/G)TCCAC	243	58	Rinttila et al., 2004

2.9. Statistical Analysis

A piglet was considered as the experimental unit for all the measurements for statistical analyses. All the data were analyzed using the general linear model (GLM) procedure of the Statistical Analysis System (SAS 8.4 SAS Institute Inc., Cary, NC, USA). One-way analysis of variance (one-way ANOVA) followed by Duncan's multiple comparison test was used to evaluate different means among treatments. The χ^2 test was used to test for diarrhea rate. Data were expressed as means ± standard error of the mean (SEM). Level of significance was set at $p < 0.05$, whereas $0.05 < p < 0.1$ was considered a trend towards significance.

3. Results

3.1. Growth Performance and Diarrhea

The growth performance and diarrhea situation of piglets are shown in Table 2. No difference was observed in the initial body weight (BW) of various groups ($p = 0.9681$). Among AB, CON, and FMT groups, the weaning BW and finial BW of FMT piglets were significantly increased in comparison with CON and AB piglets, and early intervention with FMT tended to show higher ADG than that of the other two control groups during suckling period ($0.05 < p < 0.1$). During the post-weaning period, AB piglets shown lower diarrhea rate and diarrhea index than that of CON and FMT piglets, and there was no significant difference of ADG, average daily feed intake (ADFI), and F:G among those three groups.

Table 2. Effects of early intervention on growth performance of piglets during suckling and post-weaning period.

Phases	Item	Treatments [1]						SEM	*p*-Value
		Control Groups			Treatment Groups				
		AB	CON	FMT	FMT+C	FMT+S	FMT+C+S		
Suckling period (Days 1–27)	Piglets	132	84	157	149	112	106		
	Sows	13	10	15	15	12	12		
	BW [2], kg								
	Day 1	1.55	1.57	1.55	1.53	1.55	1.54	0.01	0.9681
	Day 7	2.52	2.46	2.57	2.48	2.6	2.57	0.02	0.2404
	Day 14	4.11 ^{a,b}	4.01 ^{b}	4.31 ^{a}	4.17 ^{a,b}	4.25 ^{a}	4.26 ^{a}	0.03	0.0762
	Day 21	5.59 ^{b,c}	5.51 ^{c}	5.87 ^{a,b}	5.76 ^{b,c}	5.83 ^{a,b}	6.11 ^{a}	0.03	0.0017
	Day 27	6.88 ^{b,c}	6.85 ^{c}	7.26 ^{a,b}	6.96 ^{b,c}	7.14 ^{a,b,c}	7.51 ^{a}	0.04	0.0028
	ADG [3], g/d								
	Days 1–7	161.15	151.79	164	156.9	167.71	162.63	1.74	0.3105
	Days 8–14	227.37 ^{b,c}	220.66 ^{c}	247.81 ^{a}	241.51 ^{a,b}	235.31 ^{a,b,c}	242.4 ^{a,b}	2.17	0.0254
	Days 15–21	211.24 ^{b}	214.13 ^{b}	223.64 ^{b}	226.26 ^{b}	226.12 ^{b}	255.46 ^{a}	2.56	0.0007
	Days 22–27	214.80 ^{b,c}	223.29 ^{a,b,c}	230.57 ^{a,b}	200.49 ^{c}	219.17 ^{b,c}	243.40 ^{a}	2.62	0.0030
	Days 1–27	204.85 ^{b}	203.62 ^{b}	217.99 ^{a,b}	208.42 ^{b}	213.51 ^{b}	227.74 ^{a}	1.39	0.0032
	Diarrhea rate, %								
	Days 1–7	10.41	13.97	7.89	7.34	11.21	5.93	1.11	0.3757
	Days 8–14	5.14 ^{a}	4.27 ^{a,b}	2.81 ^{b,c}	1.98 ^{c}	3.76 ^{a,b,c}	1.96 ^{c}	0.33	0.0182
	Days 15–21	8.95 ^{a,b}	11.66 ^{a}	7.53 ^{b}	6.27 ^{b}	7.45 ^{b}	5.14 ^{b}	0.58	0.0144
	Days 22–27	4.93	4.93	4.93	4.93	4.93	4.93	0.35	0.6937
	Days 1–27	7.47 ^{a,b}	9.33 ^{a}	5.92 ^{b,c}	5.16 ^{b,c}	7.08 ^{a,b,c}	4.49 ^{c}	0.38	0.0047
	Diarrhea index								
	Days 1–7	0.52	0.6	0.47	0.47	0.58	0.46	0.09	0.9979
	Days 8–14	0.11 ^{a}	0.1 ^{a,b}	0.07 ^{a,b}	0.06 ^{a,b}	0.09 ^{a,b}	0.05 ^{b}	0.01	0.0830
	Days 15–21	0.17 ^{b}	0.27 ^{a}	0.18 ^{b}	0.15 ^{b}	0.17 ^{b}	0.12 ^{b}	0.01	0.0202
	Days 22–27	0.12	0.16	0.13	0.13	0.15	0.14	0.01	0.8997
	Days 1–27	0.23	0.29	0.22	0.21	0.25	0.19	0.03	0.9388
Post-weaning period (Days 28–63)	Piglets	81	71	83	83	84	78		
	Pans	4	4	4	4	4	4		
	BW, kg								
	Day 27	6.79 ^{c}	7.25 ^{b}	7.78 ^{a}	7.17 ^{b}	7.37 ^{b}	7.83 ^{a}	0.06	<0.0001
	Day 35	7.47 ^{d}	7.89 ^{c}	8.30 ^{b}	7.79 ^{c}	7.94 ^{c}	8.49 ^{a}	0.06	<0.0001
	Day 42	10.27 ^{c}	10.75 ^{b,c}	11.13 ^{b}	10.96 ^{b}	10.65 ^{b,c}	11.79 ^{c}	0.08	<0.0001
	Day 49	13.87 ^{d}	14.45 ^{c}	15.26 ^{b}	14.67 ^{c}	14.46 ^{c}	16.12 ^{a}	0.10	<0.0001
	Day 56	18.60 ^{d}	18.92 ^{c,d}	19.62 ^{b}	19.34 ^{b,c}	18.95 ^{c,d}	21.01 ^{a}	0.12	<0.0001
	Day 63	23.66 ^{c}	23.92 ^{c}	25.07 ^{b}	24.36 ^{b,c}	23.79 ^{c}	26.54 ^{a}	0.15	<0.0001
	ADG, g/d								
	Days 27–35	96.33	90.54	73.79	89.02	80.64	94.49	3.53	0.3333
	Days 36–42	400.77 ^{b}	405.71 ^{b}	405.71 ^{b}	456.10 ^{a}	393.00 ^{b}	462.54 ^{a}	4.80	<0.0001
	Days 43–49	503.09 ^{d}	529.34 ^{c,d}	587.14 ^{a,b}	527.21 ^{c,d}	552.67 ^{b,c}	617.25 ^{a}	5.86	<0.0001
	Days 50–56	682.17 ^{a,b}	634.53 ^{b,c}	622.79 ^{c}	667.30 ^{a,b,c}	642.52 ^{b,c}	698.55 ^{a}	6.54	0.0254
	Days 57–63	723.05 ^{a,b}	726.24 ^{a,b}	777.62 ^{a}	717.23 ^{a,b}	687.31 ^{b}	789.36 ^{a}	8.73	0.0211
	Days 27–63	481.27 ^{b}	476.74 ^{b}	494.37 ^{b}	490.71 ^{b}	471.06 ^{b}	530.74 ^{a}	3.47	<0.0001
	ADFI [4], g/d								
	Days 27–35	148.91	153.47	146.9	147.9	134.95	143.56	2.36	0.3301
	Days 36–42	466.58	472.92	475.98	486.33	488.87	520.17	6.7	0.2404
	Days 43–49	718.82	759.39	776.59	766.47	756.98	847.84	13.22	0.1060
	Days 50–56	966.53	937.12	956.86	967.15	929.34	1031.81	14.42	0.4080

Table 2. *Cont.*

Phases	Item	Treatments [1]						SEM	*p*-Value
		Control Groups			Treatment Groups				
		AB	CON	FMT	FMT+C	FMT+S	FMT+C+S		
	Days 57–63	1155.95 ^{b,c}	1176.29 ^{b,c}	1237.52 ^{a,b}	1150.79 ^{b,c}	1108.41 ^{c}	1290.33 ^{a}	17.98	0.0235
	Days 27–63	693.72	701.96	720.38	707.02	687.47	769.76	9.52	0.1284
	Diarrhea rate, %								
	Days 27–35	6.35 ^{b}	9.78 ^{a,b}	12.16 ^{a}	7.25 ^{b}	9.69 ^{a,b}	5.89 ^{b}	0.64	0.0209
	Days 36–42	3.48 ^{a}	6.60 ^{a}	6.12 ^{a,b}	4.59 ^{b,c}	4.30 ^{b,c}	2.59 ^{c}	0.33	0.0007
	Days 43–49	4.34 ^{a,b}	5.87 ^{a}	3.48 ^{a,b}	3.04 ^{b}	4.48 ^{a,b}	2.04 ^{b}	0.36	0.0374
	Days 50–56	2.21 ^{b,c}	5.56 ^{a}	3.53 ^{b}	1.99 ^{b,c}	2.41 ^{b,c}	1.12 ^{c}	0.3	<0.0001
	Days 57–63	2.08 ^{b}	5.56 ^{a}	4.42 ^{a}	1.03 ^{b}	2.41 ^{b}	1.05 ^{b}	0.37	<0.0001
	Days 27–63	3.75 ^{c,d}	6.67 ^{a}	5.96 ^{a,b}	3.69 ^{c,d}	4.67 ^{b,c}	2.60 ^{d}	0.23	<0.0001
	Diarrhea index								
	Days 27–35	0.17	0.24	0.29	0.22	0.27	0.18	0.02	0.1802
	Days 36–42	0.10 ^{b,c}	0.17 ^{a}	0.15 ^{a,b}	0.12 ^{b,c}	0.13 ^{a,b,c}	0.08 ^{c}	0.01	0.0039
	Days 43–49	0.10 ^{a,b}	0.13 ^{a}	0.09 ^{a,b}	0.08 ^{a,b}	0.12 ^{a}	0.05 ^{b}	0.01	0.0367
	Days 50–56	0.06 ^{b,c}	0.12 ^{a}	0.08 ^{b}	0.04 ^{c}	0.06 ^{b,c}	0.04 ^{c}	0.01	0.0002
	Days 57–63	0.05 ^{b}	0.13 ^{a}	0.11 ^{a}	0.03 ^{b}	0.06 ^{b}	0.03 ^{b}	0.01	<0.0001
	Days 27–63	0.10 ^{c,d}	0.16 ^{a}	0.14 ^{a,b}	0.10 ^{b,c,d}	0.13 ^{a,b,c}	0.08 ^{d}	0.01	0.0003

[1] AB = antibiotics control group; CON = placebo control group; FMT = fecal microbial transplantation control group; FMT+C = FMT added *C. butyricum*; FMT+S = FMT added *S.boulardii*; FMT+C+S = FMT added *C. butyricum* and *S.boulardii*; [2] BW=body weight; [3] ADG = average daily gain; [4] ADFI = average daily feed intake ^{a,b,c} Values within a row with different superscripts differ significantly at $p < 0.05$, All values are presented as means ± SEM.

When compared with the CON group, FMT+C+S significantly increased the weaning and finial BW and ADG, and significantly reduced the diarrhea rate during the suckling and post-weaning period. However, FMT+C and FMT+S showed no significant effect on growth performance in comparison to CON piglets. FMT+C+S treated piglets showed a significant increase of weaning and finial BW and ADG, and a significant decrease of the diarrhea rate during suckling and post-weaning period in comparison to AB-treated piglets. Moreover, when compared with FMT piglets, early intervention with FMT+C+S can also improve the weaning and finial BW and ADG, and reduce the diarrhea rate.

3.2. Biomarkers of Intestinal Barrier Function

The biomarkers of intestinal barrier function are shown in Table 3. Among AB, CON, and FMT groups, the FMT-treated significantly increased the concentration of fecal MUC2 at the age of 14 d, 21 d, 35 d, and 56 d, and reduced the level of plasma DAO and D-LA at the age of 21 d, 35 d, and 56 d. Among FMT+C, FMT+S, and FMT+C+S groups, FMT+C+S piglets shown the lowest plasma DAO and D-LA, and the highest fecal MUC2 at the age of 14 d, 21 d, 35 d, and 56 d. In addition, early intervention with FMT+C+S can also significantly increase fecal MUC2 and decrease plasma DAO and D-LA at the age of 14 d, 21 d, 35 d, and 56 d, when compared to those of CON, AB, and FMT piglets. FMT+C and FMT+S piglets shown lower plasma DAO and D-LA (at the age of 21 d, 35 d, and 56 d) and higher MUC2 (at age of 14d, 21d, 35d, and 56d) than that of AB and CON piglets. There was no significant difference of those biomarkers when compared FMT+C and FMT+S piglets to FMT piglets.

3.3. Fecal Short-Chain Fatty Acids and Fecal Succinate

To evaluate the early intervention effects on bacterial metabolites, the levels of fecal SCFAs and succinate were determined (Figure 1). Compared with the CON or AB piglets, the FMT+C+S treated piglets significantly increased the concentration of acetic acid, propionic acid, valeric acid, and total SCFAs (Figure 1A) in fecal at age of 14d of piglets. There was no significant difference of SCFAs in fecal matter at the age of 21 d and 35 d (Figure 1B,C). FMT+C, FMT+S, and FMT+C+S piglets shown a significant increase of fecal isobutyric acid, isovaleric acid, and total branched chain fatty acids (BCFAs) at the age of 56 d, in comparison with the CON piglets. Fecal succinate concentration was increased at the age of 14, 21, 35, and 56 days when compared FMT+C+S piglets to AB piglets and CON piglets, and it has no significant difference between FMT+C+S and FMT. FMT+C and FMT+S piglets had

greater fecal succinate concentration at age of 14d, 35d, and 56d in comparison to AB or CON piglets (Figure 1E).

Table 3. Effects of early intervention on the intestinal barrier function in piglets.

Item	Treatment						SEM	*p*-Value
	Control Groups			Treatment Groups				
	AB	CON	FMT	FMT+C	FMT+S	FMT+C+S		
Day 14								
Plasma DAO, ng/mL	207.2 ^{a}	190.38 ^{a,b}	193.89 ^{a,b}	173.05 ^{b,c}	189.81 ^{a,b}	161.41 ^{c}	3.34	0.0007
Plasma D-LA, umol/mL	180.08 ^{a,b}	149.35 ^{b,c}	208.71 ^{a}	147.14 ^{b,c}	204.02 ^{a}	122.80 ^{c}	8.04	0.0079
Fecal MUC2, ng/g	268.07^{b}	322.24 ^{b}	443.13 ^{a}	455.90 ^{a}	412.03 ^{a}	479.72 ^{a}	15.18	<0.0001
Day 21								
Plasma DAO, ng/mL	175.87 ^{a,b}	194.63 ^{a}	162.95 ^{b}	165.39 ^{b}	124.43 ^{c}	118.29 ^{c}	6.29	<0.0001
Plasma D-LA, umol/mL	389.47 ^{a,b}	438.17 ^{a}	343.40 ^{b}	268.90 ^{c}	222.59 ^{c}	229.70 ^{c}	18.19	<0.0001
Fecal MUC2, ng/g	237.70^{c}	301.31 ^{b}	375.16 ^{a}	425.41 ^{a}	425.37 ^{a}	416.82 ^{a}	16.24	<0.0001
Day 35								
Plasma DAO, ng/mL	166.59 ^{a}	177.94 ^{a}	102.02 ^{d}	152.17 ^{b}	130.41 ^{c}	103.81 ^{d}	3.85	<0.0001
Plasma D-LA, umol/mL	335.19 ^{a}	360.20 ^{a}	184.47 ^{d}	302.31 ^{b}	251.76 ^{c}	190.56 ^{d}	9.20	<0.0001
Fecal MUC2, ng/g	197.46 ^{d}	343.40 ^{c}	472.95 ^{a}	414.30 ^{b}	398.62 ^{b}	489.34 ^{a}	12.94	<0.0001
Day 56								
Plasma DAO, ng/mL	179.73 ^{a}	153.02 ^{b}	146.03 ^{b}	117.26 ^{c}	94.41 ^{d}	92.58 ^{d}	4.09	<0.0001
Plasma D-LA, umol/mL	338.88 ^{a}	326.82 ^{a}	272.74 ^{b}	242.06 ^{c}	166.48 ^{d}	178.75 ^{d}	8.66	<0.0001
Fecal MUC2, ng/g	221.01 ^{d}	263.35 ^{c}	364.45 ^{a}	318.08 ^{b}	310.81 ^{b}	371.43 ^{a}	8.47	<0.0001

^{a,b,c,d} Values within a row with different superscripts differ significantly at $p < 0.05$. All values are presented as means ± SEM (n = 12).

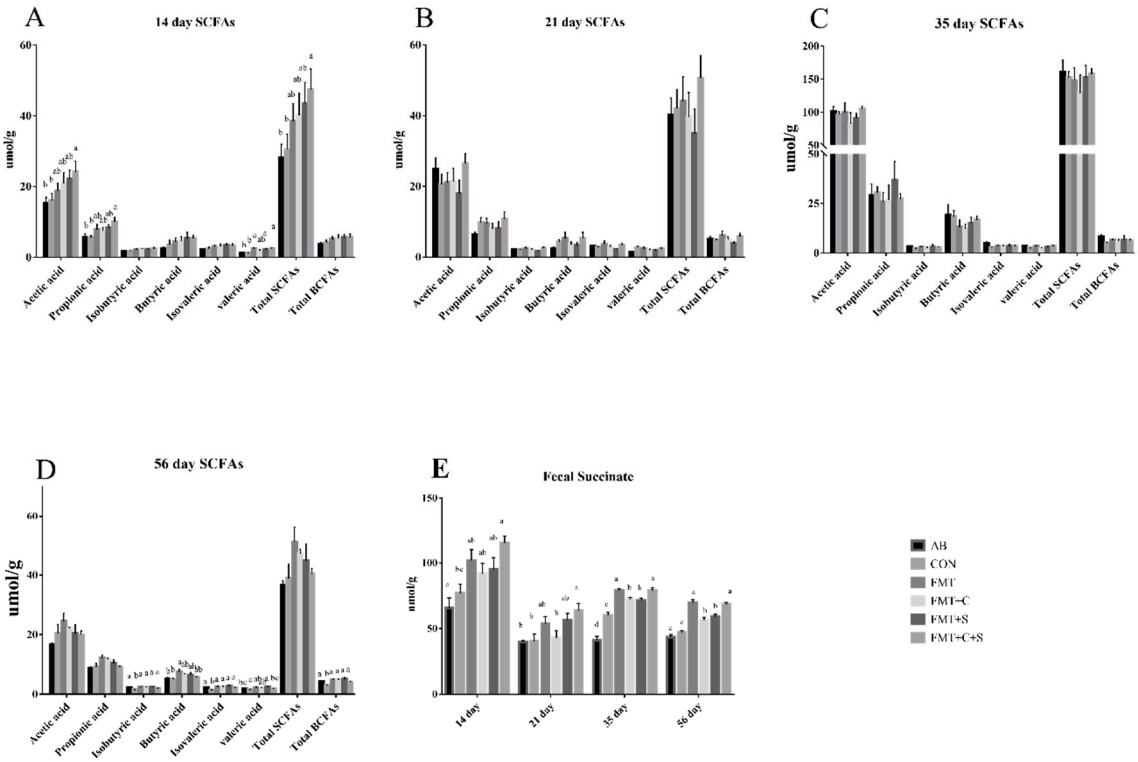

Figure 1. Effects of different early intervention model on the fecal bacterial metabolites in piglets. Fecal short-chain fatty acid (SCFA) concentrations of piglets at the age of 14 d, 21 d, 35 d and 56 d (**A**–**D**) were determined, Succinate concentration in fecal at the age of 14 d, 21 d, 35 d and 56 d (**E**). All values are presented as means ± standard error of the mean (SEM, n = 10–12). ^{a,b,c} Values within a row with different superscripts differ significantly at $p < 0.05$.

3.4. Selected Fecal Bacterial Populations in Suckling Period

As shown in Figure 2, compared with the CON piglets, early intervention with FMT+C+S significantly reduced fecal *Escherichia coli* (*E. coli*) abundance during the suckling period, and significantly increased the abundance of *Lactobacillus* spp. in piglets at the age of 3 d, 14 d and 21 d, and the FMT+C+S treatment significantly increased the abundance of *Bifidobacterium genus* in piglets at the age of 7 d, 14 d, and 21 d. When compared with AB piglets, fecal *E. coli* was decreased in FMT+C, FMT+S and FMT+C+S treated piglets at the age of 21 d. *Lactobacillus* spp. and *Bifidobacterium genus* were increased during suckling period in FMT+C+S piglets. Compared with FMT piglets, no difference was determined of fecal *E. coli* abundance in FMT+C, FMT+S and FMT+C+S piglets. The FMT+C+S treatment significantly increased the abundance of *Lactobacillus* spp. in piglets at the age of 3 d and 21 d. The FMT+C+S treatment as well as significantly increased the abundance of *Bifidobacterium* genus in piglets at the age of 7 d and 14 d.

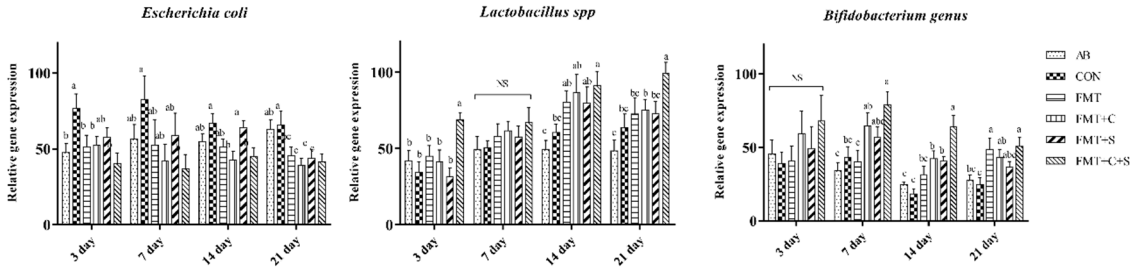

Figure 2. Effects of different early intervention model on the intestinal microbiota structure in piglets. All values are presented as means ± SEM (n = 10–12). ^{a,b,c} Values within a row with different superscripts differ significantly at $p < 0.05$.

4. Discussion

Previous studies indicated that early intervention with fecal microbiota has beneficial effects on improving growth performance and reducing the diarrhea rate in suckling piglets [2,7]. In this study, we performed an early intervention trial, and try to apply the method of early intervention to actual production. According to the results, early intervention with FMT can increase the growth performance and decrease the diarrhea rate, which is similar to previous studies [1,2,7,10]. Furthermore, we also found that the piglets' growth performance can be further improved by early intervention using FMT combined with CB and SB on the basis of FMT, which consistent with our experimental hypothesis.

To explore the reasons why FMT+C+S promoted piglets' growth performance, we determined the concentration of fecal SCFAs at different time points. Fecal SCFAs are produced by commensal bacterial fermenting indigestible dietary fibers in intestines [25,26] and participate in innate and adaptive immune responses [27,28]. In this study, the total SCFAs were increased during suckling period, after early intervention with FMT+C+S. The results indicated that early intervention with FMT+C+S may mainly enrich the SCFA produced bacteria. Emerging data discovered that succinate plays an important role in type 2 innate lymphoid cells (ILC2) immune response in the small intestine [29]. In addition, succinate levels were increased in GF mice reconstituted with the fecal microbiota of mice, and it is beneficial to protect against colonization by bacterial pathogens. Furthermore, administration of succinate in drinking water reduced colonization of bacterial pathogens [8]. Hence, in this study, the increased fecal SCFAs and succinate concentration may contribute to gain a healthier and more stable intestinal environment in FMT+C+S piglets. On the other hand, SCFAs could stimulate intestinal growth and improve gut barrier function in pig [30].

In animals, plasma DAO and D-LA were biomarkers for the functional status of intestinal mucosal barrier [31–33]. The mucus layer plays a vital in the modulation of the development and establishment of the gastrointestinal microbiota [34]. MUC2 is the most abundant mucin in the intestine and it has been used as a biomarker for gastrointestinal functionality [34,35]. The higher the level of DAO,

the more imperfect the intestinal mucosa becomes. So, our results indicated that early intervention with FMT+C+S can improve the gut barrier function.

Furthermore, we quantified several representative commensal bacteria. *Escherichia coli* is a kind of facultative anaerobic bacteria, which is planted in large quantities around 3 days after birth. Reducing the abundance of *E. coli* in piglets at the age of 14 d and 21 d, which can effectively reduce the risk of diarrhea. *E. coli* is one of Enterobacteriaceae, for which gut oxygen is a limiting resource, and the gut oxygen increases the relative abundance of Enterobacteriaceae [36], which may indicate a lower oxygen content in the gut of piglets in group FMT+C+S. *Lactobacillus* spp. are important members of the commensal microbiota and are used as probiotics. The *Lactobacillus* species were sensitivity toward hydrogen sulfide [37], and hydrogen sulfide was reported to be involved in intestinal inflammation [38]. It has been confirmed that early intervention could enrich the *Lactobacillus* spp. population [7,39]. Therefore, the increased abundance of *Lactobacillus* spp. in our study suggests that early intervention with FMT+C+S can reduce the risk of bowel disorders. *Bifidobacterium* genus have been shown to promote immune function [40–42]. The increase of *Bifidobacterium* genus during suckling period after early intervention with FMT+C+S may help for the development of the immune system of piglets. However, we did not slaughter animals to direct the detection of oxygen in the intestines in this study, therefore, further research is still needed.

It is worth noting that, in our study, antibiotics (AB group) has no effect on growth performance compared with group CON, and the diarrhea rate of piglets in group AB maintained the same high level as the CON, this result is seemingly contrary to the effect that antibiotics promote growth [43]. That may be because early antibiotic exposure can damage the infant gut microbiota, which is harmful to health [44–46]. Furthermore, research of antibiotic growth promoters has mainly been on weaning piglets, but there are few studies on suckling piglets. According to the results, antibiotics may not be necessary for suckling piglets.

5. Conclusions

This study for the first time builds an early intervention model of FMT combined with probiotics to provide colonization conditions for mature maternal fecal microbiota and anaerobic probiotic through SB's early consumption of intestinal oxygen. On the basis of FMT, FMT+C+S can further improve the growth performance and intestinal barrier function and reduce the diarrhea rate. Moreover, this study attempts to explain the mechanism of action, and the main reason is considered to be that early intervention with FMT+C+S improves the abundance of beneficial bacteria and promotes the production of fecal SCFAs and succinate. However, the underlying mechanisms need to be further explored in the future. In addition, according to the results, we have found that early antibiotic health care has no significant beneficial effect on newborn piglets.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/2/568/s1: Table S1: Information of donor sows; Table S2: Parity and litter size of experimental sows.

Author Contributions: The author' contributions are as follows: J.P. and H.W. were in charge of the whole trial; Q.X. analyzed experimental data and wrote the manuscript; Q.X., X.W., Y.P., L.W., Y.G., and C.C. for animal feeding and sample collections; Q.X., L.H., and L.Z. assisted with laboratory analyses. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: There is no conflict of interest.

Abbreviations

FMT	Fecal Microbiota Transplantation
C. butyricum	*Clostridium butyricum*
S. boulardii	*Saccharomyces boulardii*
BW	body weight
ADG	average daily weight gain
ADFI	average daily feed intake
F:G	the ratio of ADFI and ADG
SCFAs	short-chain fatty acids
DAO	diamine oxidase
D-LA	D-lactate

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