https://doi.org/10.17221/121/2017-CJAS

Effect of Supplementation with Two Combinations of Alternative to Antimicrobials by Stages on Cecal Fermentation in Rabbits

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ABSTRACT

Liu L., Xu Y., Xu X. (2018): **Effect of supplementation with two combinations of alternative to antimicrobials by stages on cecal fermentation in rabbits**. Czech J. Anim. Sci., 63, 419–427.

Antimicrobials inhibit cecal fermentation when preventing rabbit from infection. This study aimed to evaluate the effect of supplementation with two combinations of alternative to antimicrobial (combination I: 1×10^9 cfu/kg *Bacillus subtilis* + 2 g/kg fructooligosaccharide; combination II: 2 g/kg acidifier and 0.6 g/kg essential oil) by stages on rabbit's growth performance and cecal fermentation. Two hundred and forty 15-day-old male kits with similar body weight were distributed randomly to five groups, which were control (basal diet), ZnB (addition of 0.1 g/kg bacitracin zinc in basal diet), II (addition of combination II), I-II (addition of combination I during days 15–35, addition of combination II during days 36–77), and I-II-I (supplemented with combination I during days 15–35 and 57–77, supplemented with combination II during days 36–56). Each group had 6 replicates. One healthy rabbit from each replicate was slaughtered at day 35 and day 77. The results showed: (1) at day 35, the two combinations and bacitracin zinc all inhibited ileal *Escherichia coli* ($P < 0.05$), decreased cecal pH, and increased total volatile fatty acid concentration ($P < 0.05$). Combination I decreased duodenal crypt depth and increased duodenal villi height to crypt depth ratio (VCR) ($P < 0.05$); (2) at day 77, I-II-I group had more cecal total bacteria than control ($P < 0.05$). Mode I-II or I-II-I increased cecal *Bacteroides-Prevotella* ($P < 0.05$) compared with ZnB. Mode I-II-I shortened duodenal crypt depth and increased VCR compared with control or ZnB ($P < 0.05$); (3) after weaning, modes I-II-I and I-II had better or similar effect on decreasing diarrhoea and mortality rate compared with ZnB. In conclusion, both modes had better or similar effect on decreasing diarrhoea and mortality rate compared with inclusion of antimicrobial or combination II alone during the whole trial, and mode I-II-I showed better effect than mode I-II.

Keywords: *Bacillus subtilis*; acidifier; essential oil; fructooligosaccharide; fermentation trait

Because of undeveloped gut immunity system, growing rabbit is susceptible to intestinal infection, which often leads to serious gut diseases and subsequent death, especially in 2–3 weeks after weaning (Fortunlamothe and Boullier 2007). To prevent rabbits from pathogen infection, antibiotic is generally added in rabbit's diet, but rabbit is herbivore with simple stomach and big cecum, which occupies 40–60% of the total volume of the gastrointestinal tract (Jenkins et al. 2000), and the by-products of cecal fermentation provide about 40% maintenance energy requirement for rabbit (Marty and Vernay 1984) and microbial protein synthesized in cecum represents about 10% of total

Supported by the Key Science and Technology Program of Shaanxi Province (No. 2013K02-18).

L. Liu and Y. Xu contributed equally to this work.

https://doi.org/10.17221/121/2017-CJAS

daily protein intake (de Blas and Mateos 2010). Therefore, except for the well-known security issue, inclusion of antimicrobial in rabbit's diet can inhibit cecal fermentation and then decrease rabbit's feed efficiency. Thus, it is necessary to use alternatives to antibiotic in rabbit farming in order to maintain the normal fermentation in cecum when preventing rabbit from pathogenic infection. Our previous studies showed that supplementation with acidifier (Zhu et al. 2014) or probiotics (unpublished) alone had no obvious effect on decreasing rabbit's mortality and diarrhea rate in newly weaned rabbits. We hypothesized that combining different alternatives together according to their main function and supplementing them to rabbits in stages may not inhibit the cecal fermentation while preventing rabbits from pathogenic infection.

Bacteroides is predominant in rabbit's intestine after supplementation with solid food (Fekete 1989; FortunLamothe and Boullier 2007). It was reported that *Bacteroides fragilis* stimulated the development of rabbit's gut associated immunity system when injected with *Bacillus subtilis* (*B. subtilis*) in rabbit's vermiform appendix (Rhee et al. 2004; Hanson and Lanning 2008). We found that oral administering the rabbit with *Bacteroides fragilis* could increase intestinal *Bacteroides fragilis* population (unpublished). But as a strict anaerobe, *Bacteroides fragilis* is inappropriate for being developed as rabbit's probiotics. Experimental results from both *in vivo* and *in vitro* suggested that fructooligosaccharide can promote the proliferation of *Bacteroides fragilis* (Euler et al. 2005; Kapiki et al. 2007; Mao et al. 2015). Therefore, the present study aimed to combine fructooligosaccharide and *Bacillus subtilis* as a stimulatory combination that may stimulate the proliferation of *Bacteroides* in rabbit's gastrointestinal tract and promote the development of gut associated immune system.

Acidifier and essential oil have antibacterial activity to both Gram-positive and Gram-negative bacteria (Si et al. 2006) and are widely used to inhibit intestinal pathogen in animal farming (Maenner et al. 2011; Hashemi et al. 2012; Romero et al. 2012; Gopi et al. 2014). These two antibacterial substances can be absorbed in animal's foregut, therefore, if they were combined into an inhibitory combination and added in rabbit's diet, they may not seriously inhibit the fermentation in rabbit's cecum when effectively preventing rabbit from infection.

In order to promote the development of rabbit's intestinal microflora before weaning and prevent the intestinal pathogen effectively in newly weaned rabbits but not inhibit cecal fermentation in growing ones, the present study designed two modes to supply the two combinations to rabbit in stages, and compared their effects on rabbit's growth performance and cecal fermentation with negative control, bacitracin zinc and the inhibitory combination. We did not detect the stimulatory combination alone during the entire fattening period because it had already been detected in a prior study and currently not enough eligible rabbits were at disposal.

MATERIAL AND METHODS

Animals and experimental design. The use of animals and the experimental procedure followed the Guide for the Care and Use of Laboratory Animals of the National Institute of Health and were approved by the Animal Care and Use Committee of Northwest A&F University, China.

Two hundred and forty healthy male kits of Ira rabbit were selected based on age and body weight at 15 days of age from the reproduction stock of a commercial farm and randomly distributed into 5 groups with 6 replicates per group and 8 rabbits per replicate. The five groups were: control (rabbits were fed with the basal diet without antimicrobial or alternatives to antimicrobial, and this group served as the negative control), ZnB (rabbits were supplemented with 0.01 g/kg bacitracin zinc in the basal diet, this group served as the positive control), II (rabbits were supplemented with combination II in the basal diet during the whole feeding period), I-II (rabbits were supplemented with combination I in the basal diet before weaning, but supplemented with combination II in the basal diet after weaning), and I-II-I (animals were only supplemented with combination I in the basal diet before weaning and during 57–77 days of age, but only supplemented with combination II during 36–56 days of age). The diet of each group at different stage is presented in Table 1. Combination I (the stimulatory combination) was composed of *B. subtilis* (1×10^9 cfu/kg in basal diet) and fructooligosaccharide (0.2 g/kg in basal diet), and combination II (the inhibitory combination) included acidifier (containing a

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Table 1. Details of the experimental design

Group	Age (days)		
	15–35	36–56	57–77
Control	basal diet (BD)		
ZnB	BD + 0.1‰ bacitracin zinc		
II	BD + combination II		
I-II	BD + combination I	BD + combination II	
I-II-I	BD + combination I	BD + combination II	BD + combination I

combination I: 1 × 10^9 cfu/kg *B. subtilis* + 2 g/kg fructooligosaccharide; combination II: 2 g/kg acidifier and 0.6 g/kg essential oil

mixture of formic acid, acetic acid and ammonium formate, 0.2 g/kg in basal diet) and essential oil (containing a mixture of thyme and thymol oil, 0.06 g/kg in basal diet). Suckling rabbits were supplied with solid feed from 15 days of age. The feeding experiment ended at 77 days of age. All the groups were weaned at 35 days of age.

The basal diet was designed according to NRC (1977) (Table 2). Rabbit was kept in standard cage in an automatic building with temperature between 25 and 26°C, and a natural photoperiod (about 12–14 h light and 10–12 h dark) was provided during the entire feeding experiment. Except for suckling time, rabbits were separated from their mother from the first day of the experiment.

From 15 days of age, live weight and feed intake were recorded weekly before morning feeding, and mortality and diarrhea were assessed every morning. Average daily gain (ADG), mortality rate, and diarrhea rate were evaluated during the first stage (15–35 days of age) and the second stage (36–77 days of age). Average daily feed intake (ADFI) and feed/gain ratio (F/G) were calculated only for the second stage. At 35 and 77 days of age, one healthy rabbit from each replicate (6 rabbits from each treatment), whose body weight was close to the average weight of a replicate, was slaughtered. Duodenal tissues were fixed in 10% neutral formalin for histological examination, and ileal and cecal content was collected for determination of bacterial number and cecal volatile fatty acid (VFA) and ammonia N concentration.

Histological examination. One segment of duodenum, which was about 2.5 cm in length, was cut longitudinally at the mesenteric attachment and immediately fixed in 10% neutral formalin after washing with sterile saline. The measurements were determined according to a modified method of Iji et al. (2001). Crypt depth (CD) and villi height (VH) were observed using a phase contrast microscope (Biomedica Magnoni S.n.C., Nikon Instruments S.p.A., Italy). Three sections were prepared for each sample, and 3 horizons for each section were selected. CD and VH were determined using the average of the nine measurements, and the value of duodenal villi height to crypt depth ratio (VCR) was calculated.

Measurement of cecal pH, VFA, and ammonia N concentration. Immediately after slaughter, the pH of cecal content was measured using a glass electrode pH meter (CT-3031; Shenzhen Kedida Electronics Co., Ltd., China). Digesta content from each rabbit was divided into three equal subsamples. One subsample was used for bacterial analysis, the other two were immediately stored at –20°C for measuring cecal VFA and ammonia N (NH_3-N) concentration, respectively. VFA concentration was determined by a high performance

Table 2. Composition and nutrition levels of basal diet (air-dry basis) (%)

Ingredients	Content (%)	Nutrition level[b]	Content (%)
Alfalfa meal	32.04	dry matter	87.30
Corn	31.99	crude protein	16.50
Soybean meal	13.05	NDF	30.00
Wheat bran	20.00	DE (kcal/kg)	2505
Salt	0.40	Ca	0.85
Limestone	0.20	total phosphorus	0.62
DL-Methionine	0.36	lysine	0.85
Lysine	0.16	methionine	1.31
Calcium hydrophosphate	1.31	threonine	0.80
Threonine	0.25		
Minerals and vitamins premix[a]	0.24		

NDF = neutral detergent fibre, DE = digestible energy

[a]premix provided per kg of diet: vitamin A 12 000 IU, vitamin D3 2500 IU, vitamin E 40 mg, vitamin K 2.0 mg, vitamin B1 2.0 mg, vitamin B2 4 mg, vitamin B6 2.0 mg, vitamin B12 0.01 mg, biotin 0.06 mg, niacin 50 mg, folic acid 0.3 mg, D-pantothenic acid 10 mg, choline 1000 mg, Zn 40 mg, Cu 10 mg, Mn 30 mg, Fe 50 mg, I 0.5 mg, Se 0.2 mg, Co 0.5 mg

[b]value was calculated based on the feed manufacturer's information

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Table 3. Specific primers of total bacteria, *Bacteroides-Prevotella*, and *Escherichia coli*

Item	Primer	Primer sequence (5'–3')	Amplicon size (bp)
Total bacteria	1114f	CGGCAACGAGCGCAACCC	130
	1275r	CCATTGTAGCACGTGTGTAGCC	
Bacteroides-Prevotella	forward	AACGCTAGCTACAGGCTT	272
	reverse	CCAATGTGGGGGACCTTC	
Escherichia coli	forward	GTTAATACCTTTGCTCATTGA	340
	reverse	ACCAGGGTATCTAATCCTGTT	

liquid chromatograph (L-2000 Series LaChrom Elite; Hitachi, Japan) according to Zhao et al. (2011). The concentrations of ammonia were assayed using the indophenol method (Chaney and Marbach 1962).

Determination of the number of ileal and cecal bacteria. Total bacterial genomic DNA from each cecal and ileal sample was extracted by a modified phenol-chloroform-isoamylalcohol extraction method (Zhu et al. 2014). Concentration of the extracted DNA solution was subsequently determined, and then diluted to a concentration of 15 ng/μl.

The number of total bacteria, *Bacteroides-Prevotella*, and *Escherichia coli* (*E. coli*) in cecal and ileal content was determined by absolute quantitative real-time PCR. Standard DNA for each bacterium was prepared by recombining the PCR fragment with the T-vector. Then the standard curve was made by quantitative real-time PCR after serial 10-fold dilutions of the standard DNA. The copy number of the specific fragment in total bacterial genomic DNA of each sample was calculated to get the number of the corresponding bacteria.

The reaction mixture (20 μl) of real-time PCR consisted of 10 μl of SYBR Premix *Ex Taq* (TaKaRa Biotechnology (Dalian) Co., Ltd, China), 0.4 μM of each primer, and 30 ng of the extracted bacterial genomic DNA. The amount of bacterial DNA in each sample was determined in triplicate, and the mean values were calculated. Real-time PCR was performed on Bio-Rad iQ5 PCR System (BioRad Laboratories Inc., USA) with an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 45 s. The primers used for detecting bacterial number are listed in Table 3 (Huijsdens et al. 2002; Denman and Mcsweeney 2006; Converse et al. 2009).

Statistical analysis. Mortality rate was analysed using the chi-square test, and other data were analysed using the one-way model of ANOVA (SPSS 19.0). Significance was declared at $P < 0.05$, and trends were discussed at $P < 0.1$.

RESULTS

Effects of treatments on rabbit's growth performance. Rabbits supplemented with combination I (*B. subtilis* and fructooligosaccharide, Table 4) before weaning tended to have higher average daily gain (ADG) ($P < 0.1$) compared with control. During

Table 4. Effects of treatments on rabbits' growth performance

Item	Days of experiment	Groups					SEM	*P*-value
		Control	ZnB	II	I-II	I-II-I		
ADG (g/day)	15–35	21.00	23.33	22.67	23.83	24.17	0.42	0.086
	36–77	33.51	34.26	33.48	34.05	35.30	0.27	0.257
ADFI (g/day)	36–77	116.46	117.80	114.49	113.61	114.60	0.54	0.189
F/G	36–77	3.48	3.44	3.43	3.34	3.25	0.03	0.077
Mortality rate (%)	15–35	8.33	4.17	2.08	4.17	0.00		0.244
	36–77	20.08^{a}	9.92^{b}	12.50^{b}	12.50^{b}	6.25^{b}		0.024
Diarrhoea rate (%)	15–35	2.81^{a}	1.93^{a}	1.52^{ab}	1.50^{ab}	1.02^{b}		0.046
	36–77	3.35^{a}	3.39^{a}	3.29^{ab}	3.11^{ab}	3.00^{b}		0.043

ADG = average daily gain, ADFI = average daily feed intake, F/G = feed/gain ratio, SEM = standard error of the mean

^{a,b}in the same row, values with different superscripts mean a significant difference ($P < 0.05$)

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Table 5. Effects of treatments on populations of ileal and cecal bacteria in rabbits at 35 days of age (10 ^ cfu/g content)

Item	Location	Groups					SEM	*P*-value
		Control	ZnB	II	I-II	I-II-I		
Total bacteria	ileum	8.27	8.32	8.62	8.41	8.54	0.10	0.620
	cecum	10.70	10.85	11.12	10.92	11.10	0.09	0.439
Escherichia coli	ileum	6.44[a]	5.67[b]	5.64[b]	5.82[b]	5.69[b]	0.12	0.042
	cecum	7.99	7.39	7.46	7.64	7.43	0.09	0.094
Bacteroides-Prevotella	ileum	7.39	7.35	7.45	7.41	7.69	0.14	0.664
	cecum	9.95	10.21	10.31	10.40	10.47	0.07	0.086

SEM = standard error of the mean

[a,b]in the same row, values with different superscripts mean a significant difference ($P < 0.05$)

36–77 days of age, rabbits in I-II-I group tended to have lower feed/gain ratio than rabbits in control, antimicrobial and II groups ($P < 0.1$). Control group had higher mortality rate than other groups ($P < 0.05$). Feeding mode I-II-I decreased diarrhoea rate significantly compared with control or antimicrobial group from 36 to 77 days of age ($P < 0.05$).

Effects of treatments on the number of intestinal bacteria. As presented in Table 5, antimicrobial, combination I and combination II all inhibited proliferation of *E. coli* in ileal content before weaning ($P < 0.05$), but they did not show a different inhibitory effect on *E. coli* ($P > 0.05$). The number of cecal *Bacteroides-Prevotella* in rabbits fed with combination I tended to be higher than that in control rabbits ($P < 0.1$).

At 77 days of age (Table 6), the number of total bacteria per g cecal content in I-II-I group was higher than that in control and antimicrobial groups ($P < 0.05$). I-II and I-II-I groups had more *Bacteroides-Prevotella* in cecal content ($P < 0.05$) compared with antimicrobial group. Rabbits in I-II-I or I-II group had higher *Bacteroides-Prevotella* population in cecal content than rabbits in II group ($P < 0.05$). There were no obvious differences in the number of total bacteria or *Bacteroides-Prevotella* in cecal content between antimicrobial and combination II groups during the whole trial ($P > 0.05$). Antimicrobial had the trend of deceasing the cecal *Bacteroides-Prevotella* population ($P < 0.1$).

Effects of the treatments on cecal fermentative traits. Rabbits in control group had higher cecal pH and NH_3-N ($P < 0.05$, Table 7) and lower total VFA (TVFA) and acetic acid concentration ($P < 0.05$) than rabbits supplemented with combination I, combination II or antimicrobial. But there was no difference in the fermentative traits (except for valeric acid) between antimicrobial group and other three groups, whose creep feed was added with combination I or II.

Results in Table 8 showed that there was no significant difference in all the investigated fermentative traits between the five groups when rabbits grew up to 77 days old. But rabbits in I-II and I-II-I groups tended to have higher TVFA than in control and antimicrobial groups ($P < 0.1$). I-II

Table 6. Effects of treatments on populations of ileal and cecal bacteria in 77-day-old rabbits (10 ^ cfu/g content)

Item	Location	Groups					SEM	*P*-value
		Control	ZnB	II	I-II	I-II-I		
Total bacteria	ileum	8.28	8.33	8.54	8.54	8.55	0.07	0.180
	cecum	10.85	10.78	11.02	11.12	11.31	0.06	0.083
Escherichia coli	ileum	6.45	6.11	5.84	5.99	5.82	0.10	0.509
	cecum	8.19	8.17	7.93	7.68	7.59	0.16	0.606
Bacteroides-Prevotella	ileum	7.34	7.30	7.42	7.68	7.73	0.07	0.187
	cecum	10.35[bc]	10.11	10.20	10.48[ab]	10.53[a]	0.05	0.018

SEM = standard error of the mean

[a,b]in the same row, values with different superscripts mean a significant difference ($P < 0.05$)

https://doi.org/10.17221/121/2017-CJAS

Table 7. Effects of treatments on cecal pH and fermentative traits (mM) of 35-day-old rabbits

Item	Groups					SEM	*P*-value
	Control	ZnB	II	I-II	I-II-I		
pH	6.37	6.04	6.02	6.03	6.02	0.06	0.099
NH_3-N	17.35^{a}	10.70^{b}	11.56^{b}	11.95^{b}	11.47^{b}	0.89	0.012
TVFA	9.47^{b}	12.38^{a}	12.66^{a}	13.24^{a}	12.99^{a}	0.63	0.014
Acetic acid	7.84^{b}	9.95^{a}	10.17^{a}	11.95^{a}	11.03^{a}	0.49	0.013
Propionic acid	0.52	0.72	0.65	0.73	0.66	0.04	0.268
Butyric acid	0.89	1.39	1.48	1.21	1.40	0.11	0.213
Valeric acid	0.15^{b}	0.20^{b}	0.29^{a}	0.31^{a}	0.30^{a}	0.02	0.001
Isovaleric acid	0.03	0.04	0.05	0.04	0.06	0.01	0.511

TVFA = total volatile fatty acid, SEM = standard error of the mean

^{a,b}in the same row, values with different superscripts mean a significant difference ($P < 0.05$)

Table 8. Effects of treatments on cecal pH and fermentative traits (mM) of 77-day-old rabbits

Item	Groups					SEM	*P*-value
	Control	ZnB	II	I-II	I-II-I		
pH	5.94	6.18	6.04	5.85	5.70	0.06	0.352
NH3-N	15.95	18.57	16.34	15.20	12.95	0.93	0.528
TVFA	19.04	19.09	18.51	21.42	23.73	0.68	0.089
Acetic acid	14.99	14.05	13.67	15.98	18.69	0.56	0.156
Propionic acid	0.95	0.97	0.92	1.16	1.19	0.05	0.229
Butyric acid	2.58	2.70	3.38	3.22	2.81	0.24	0.330
Valeric acid	0.32	0.32	0.34	0.36	0.36	0.02	0.503
Isovaleric acid	0.12	0.12	0.13	0.13	0.16	0.01	0.550

TVFA = total volatile fatty acid, SEM = standard error of the mean

group had by 16% higher TVFA in cecal content than II group ($P < 0.1$).

There were no significant effects of treatments on the proportion of each VFA to TVFA in rabbit's cecum at the age of 35 or 77 days (data not shown).

Effects of treatments on intestinal morphology of rabbit. Supplementing with combination I increased duodenal VCR in 35-day-old rabbit ($P < 0.05$) compared with control group (Table 9), but both combined II and antimicrobial had no obvious effect on this index ($P > 0.05$). At 77 days

Table 9. Effects of treatments on intestinal morphology of rabbit

Item	Day	Groups					SEM	*P*-value
		Control	ZnB	II	I-II	I-II-I		
VH (μm)	35	458.7	469.4	469.4	553.6	541.0	17.0	0.205
	77	537.7	519.6	543.3	571.1	583.4	12.2	0.717
CD (μm)	35	82.79	70.32	70.32	66.02	65.25	2.78	0.147
	77	104.34^{a}	109.19^{a}	98.35^{ab}	94.73^{ab}	85.66^{b}	2.68	0.030
VCR	35	6.04^{b}	7.01^{b}	6.81^{b}	8.40^{a}	8.35^{a}	0.32	0.026
	77	5.25^{b}	5.54^{b}	5.54^{b}	6.29^{ab}	6.89^{a}	0.24	0.027

VH = villi height, CD = crypt depth, VCR = villi height/crypt depth ratio, SEM = standard error of the mean

^{a,b}in the same row, values with different superscripts mean a significant difference ($P < 0.05$)

https://doi.org/10.17221/121/2017-CJAS

of age, rabbits in group I-II-I had obviously lower duodenal CD and higher VCR value than those in control, antibiotic and II groups ($P < 0.05$), while the difference in duodenal morphology between group I-II and control or antimicrobial or II group was not marked.

DISCUSSION

Antimicrobial can inhibit cecal fermentation and decrease rabbit's food digestion when preventing rabbit from pathogen infection. Zinc bacitracin is the most widely used antibiotic in rabbit's diet (Falcao-e-Cunha et al. 2010) and was reported to have obvious inhibition effect on intestinal *E. coli* or *Clostridium perfringens* (Romero et al. 2012) and reduce cecal bacteria population (Pinheiro et al. 2005) in growing rabbit. Like the previous reports, the present study showed that zinc bacitracin inhibited ileal *E. coli* effectively before weaning and obviously decreased morality rate after weaning, and tended to reduce *Bacteroides-Prevotella* population in the cecal content at the age of 77 days compared with control. In the present study, there was no difference in cecal *Bacteroides-Prevotella* population between control and antimicrobial group at the age of 35 days, but rabbit in control tended to have higher cecal *Bacteroides-Prevotella* population than antimicrobial group at 77 days of age. The obviously higher intestinal *E. coli* population before weaning in control group might inhibit the proliferation of *Bacteroides-Prevotella*, but when rabbit grew up to 77 days of age, cecal *Bacteroides-Prevotella* population of healthy rabbit in control group increased to normal level while the inhibitory effect of antimicrobial on *Bacteroides-Prevotella* was persistent, and therefore rabbits in control tended to have more cecal *Bacteroides-Prevotella* than rabbits in antimicrobial group.

According to their function, four different alternatives to antimicrobial were combined into two combinations and supplied to rabbit through two feeding modes. Our results indicated that both modes had a better or similar effect on decreasing rabbit's mortality rate compared with inclusion of zinc bacitracin or combination II alone throughout the entire feeding period. Mode I-II-I tended to decrease the F/G ratio during 36 to 77 days of age if compared with the supplementation with antimicrobial, combination II alone or no additive, and this decrease was consistent with higher *Bacteroides-Prevotella*, total bacteria and better intestinal morphology in I-II-I group than in the three groups at 77 days of age.

There were several reports about the positive effect of mannanoligosaccharides on the growth performance and/or cecal fermentation in weaned rabbit (Mourao et al. 2006; Guedes et al. 2009), while Pinheiro et al. (2009) found that the addition of mannanoligosaccharides to low fibre diet did not affect any cecal traits. No study has been reported about the use of fructooligosaccharide alone or with *B. subtilis* in suckling or growing rabbit till now, but evidences showed that fructooligosaccharide stimulated the proliferation of *Bacteroides-Prevotella* (Kapiki et al. 2007; Mao et al. 2015), which is the dominant bacteria in rabbit's cecum and the main by-product of its anaerobic respiration is acetic acid (Harrison and Hansen 1963). As we supposed, supplementation with fructooligosaccharide and *B. subtilis* together increased TVFA and acetic acid, and tended to increase the number of *Bacteroides-Prevotella* in cecal content compared with negative control before weaning. Inclusion of combination I alone before weaning improved the proliferation of cecal *Bacteroides-Prevotella* compared with control, antimicrobial or combination II, and this increase seemed to have a prolonged effect, because mode I-II tended to have higher cecal *Bacteroides-Prevotella* population and TVFA or acetic acid concentration at 77 days of age than the addition of combination II alone throughout the entire period. Jacquier et al. (2014) once reported that rapidly fermentable fibre stimulated cecal microbial activity in young rabbit. Here we confirmed that addition of stimulatory alternatives before weaning favoured the development of intestinal microbiota. Oso et al. (2013) found that inclusion of probiotic (*Pediococcus acidilactis* or *Bacillus cereus*) in the diet did not increase cecal TVFA concentration in rabbit, and our previous study also suggested that *B. subtilis* alone did not promote proliferation of *Bacteroides-Prevotella* (unpublished). Therefore, the stimulatory effect of combination I on *Bacteroides-Prevotella* should be attributed to fructooligosaccharide.

Newly weaned rabbit is extremely susceptible to intestinal infection. The present study did not detect the stimulatory combination after weaning because we had detected it before and we had not

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enough eligible rabbits here, but we investigated an inhibitory combination which was composed of organic acid and essential oil. Cardinali et al. (2008) once reported that integration of microincapsulated organic acids and essential oils increased intestinal non-pathogen bacteria and reduced intestinal morphological damage in rabbit experimentally infected with *Escherichia coli* and *Clostridium perfringens* compared with zinc bacitracin. Our results showed that combination II inhibited ileal or cecal *E. coli* effectively before weaning and showed similar effect on reducing mortality rate as zinc bacitracin. But supplementation with this inhibitory combination alone from 15 days of age to the end of the trial tended to inhibit cecal fermentation suggesting that the inclusion of alternatives to antibiotic with strong antimicrobial activity also suppressed the cecal microflora.

The higher cecal *Bacteroides-Prevotella* population and acetic acid concentration, and similar effect on reducing morality and diarrhea rate in I-II-I group compared with I-II group indicated that supplementation with a stimulatory combination after rabbits passing through a serious weaning stress is more scientific than persistent addition of an inhibitory one.

CONCLUSION

Combination I and combination II showed a similar inhibitory effect on the proliferation of *E. coli* compared with bacitracin zinc before weaning. Combination I tended to promote the proliferation of *Bacteroides-Prevotella*. Both modes I-II-I and I-II were effective on decreasing diarrhea and mortality rate, and the former was more effective than the later.

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Received: 2017–12–11
Accepted after corrections: 2018–07–18