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EFFECTS OF DOE-LITTER SEPARATION ON INTESTINAL BACTERIA, IMMUNE RESPONSE AND MORPHOLOGY OF SUCKLING RABBITS

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Abstract: Gut development is stimulated by exposure to microorganisms, especially early-life microbial exposure. This study aimed to investigate whether doe-litter separation, which is performed in many rabbit farms, affects this exposure and therefore inhibits the development of intestinal system in suckling rabbits. Immediately after parturition, Rex rabbit does (n=16) were adjusted to 8 kits per litter and divided into doe-litter separation (DLS) group and doe-litter together (DLT) group based on the conditions of the does. One healthy kit per litter was selected and sacrificed at 7 d, 14 d, 21 d and 28 d of age, and the number of total bacteria, *Escherichia coli* and *Bacteroides-Prevotella,* expression of interleukin 6 (IL-6) and interleukin 10 (IL-10) in duodenum and caecum were investigated by real-time polymerase chain reaction. The morphological parameters of duodenum and vermiform appendix were also measured. Our results showed that doe-litter separation affected the number of intestinal bacteria. At 7 d of age, except for caecal *Escherichia coli*, the number of the investigated bacteria was decreased by doe-litter separation ($P<0.05$). But 1 wk later, only the number of total bacteria and *Bacteroides-Prevotella* in caecal content ($P<0.05$) and *Escherichia coli* in duodenal content from DLS kits ($P<0.05$) were still lower than those from DLT kits. After being provided with supplementary food for 7 d, DLS kits had fewer total bacteria in caecal content ($P<0.05$) and fewer *E. coli* in duodenal content ($P<0.01$) than DLT kits. After growing to 28 d of age, kits in DLS group still tended to have fewer total bacteria in caecal content, and expression of IL-10 and secretion of secretory IgA (sIgA) in vermiform appendix in DLS group was obviously lower than kits in DLT group ($P<0.05$). The villus height:crypt depth ratio in duodenum at 3rd wk and 4th wk was decreased by DLS ($P<0.05$). Kits in DLS group had shorter villus height ($P<0.05$), higher crypt depth ($P<0.05$) and shorter vermiform appendix ($P<0.05$) at the end of the trial. Furthermore, separating kits from the doe had a negative effect on their average daily gain at 3rd wk and 4th wk ($P<0.05$). Limiting the microbiological contact with the mother during suckling period affected the kits' intestinal flora and could negatively affect the development of intestinal digestive and immune system and growth performance of kits.

Key Words: doe-litter separation, suckling rabbit, gut development, bacteroid, interleukin 6, sIgA.

INTRODUCTION

Due to undeveloped gut associated immune system and/or digestive systems, newly weaned rabbits are susceptible to intestinal infection, which often leads to high diarrhoea and mortality rates. Studies indicated that development of gut immune system is broadly stimulated by exposure to microorganisms (Umesaki *et al.,* 2000), especially early-life microbial exposure (Kelly, 2007), and the immune effects of early-life microbial exposure are durable and persist into later life (Ege, 2011). Furthermore, according to new research in mice, increasing exposure to germs stimulates the development of immune system (Olszak *et al.,* 2012) and lack of exposure to microbes at an early age increases susceptibility to some diseases due to immune system suppression. The gastrointestinal tract of newborn mammals is sterile (Hooper, 2004). Colonisation of microorganisms in this tract starts from birth (Fortun-Lamothe and

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Boullier, 2004), and its establishment is a slow process (Combes *et al.,* 2011) affected by many factors, including the microbiota of the dam's vagina (Mändar and Mikelsaar, 1996), the living environment and contact with mother. In order to improve the does' breeding performance and protect kits from infection by reducing contact with the doe's faeces, doe-litter separation during suckling period is performed in many rabbit farms in China. But this reduction may delay the early establishment of gut microflora and lead to a negative effect on early development of gut associated immune and/or digestive system, thus compounding the crisis encountered by the newly weaned rabbit. The aim of this study was to investigate the effect of doe-litter separation on intestinal bacteria, immune parameters and morphology of suckling kits, whose growth performance was also concerned.

MATERIALS AND METHODS

Experimental diet

The creep feed for kits based on corn, alfalfa meal and soybean meal was formulated according to Zhu *et al.* (2014). Ingredient and chemical composition of the diet are shown in Table 1. Does were fed with commercial feed (the formula was not provided).

Animals and experimental design

The use of animals and the experimental procedure followed the Guide for the Care and Use of Laboratory Animals of NIH and was approved by the Animal Care and Use Committee of Shaanxi Province, China.

Sixteen litters of neonatal commercial American Rex rabbit in a rabbit farm were selected based on the doe's condition and weight of the kits and randomly divided into 2 groups: doe-litter separation (DLS) group and doe-litter together (DLT) group. Each doe nursed 8 kits. Kits in DLS group were nursed twice every day at 8:00 am and 7:00 pm after feeding the does, and faeces produced by the doe during nursing time (6 min each time) were removed immediately after nursing. In the DLT group, does could enter the kit's cage freely except for the time when feeding kits and does, and faeces produced by does were not removed before nursing the next morning. Kits in each litter were supplied with equal creep feed (without antibiotics or probiotics) from 15 d of age to the end of the trial. The local temperature was 24-30/15-18 °C during the trial.

Table 1: Experimental diet ingredients and chemical composition.

Item	
Ingredients (g/kg feed)	
alfalfa meal	317
corn	276
soybean meal	177
wheat bran	200
premix[a]	1.5
salt	4
limestone	3.9
DL-methionine	4.1
lysine	0.5
calcium hydro phosphate	13
threonine	2
Chemical composition	
dry matter (g/kg feed)	868
crude protein (g/kg DM)	202
neutral detergent fibre (g/kg DM)	318
ether extract (g/kg DM)	30
digestible energy (MJ/kg DM)	11.89
Ca (g/kg DM)	10.4
total phosphorus (g/kg DM)	7.4
lysine (g/kg DM)	9.8
methionine+cysteine (g/kg DM)	9.3
threonine (g/kg DM)	9.2

[a]Premix provided the requirement minerals and vitamins.

Growth performance of baby rabbits

Kits were weighed individually before nursing at 7th, 14th, 21th and 28th d of age. Average daily gain (ADG) was calculated from birth to the end of the trial.

Sampling of intestinal content and tissue

One healthy kit with average weight per litter was slaughtered at 7th, 14th, 21st and 28th d of age to collect duodenum, vermiform appendix, duodenal and caecal content. One segment of each duodenum was fixed in 10% formalin at the same time. Intestinal content and the unfixed tissue samples were stored at –80°C for subsequent measurement. Length of the vermiform appendix was quickly measured from its end to the

junction of caecum and appendix before sampling. The intestinal wall thickness of vermiform appendix was measured from the tip of the crypt to the epicuticle.

Real-time polymerase chain reaction assay for determination of the number of intestinal Bacteroides-Prevotella, Escherichia coli *and total bacteria*

Total bacterial genomic DNA of intestinal content was extracted by modified phenol-chloroform-isoamyl alcohol 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 population size of *Bacteroides-Prevotella, Escherichia coli* and total bacteria in intestinal content was determined by SYBR green-based absolute quantitative real-time polymerase chain reaction (qPCR). To prepare the qPCR DNA standards, 3 general PCRs were performed using the *Bacteroides-Prevotella, Escherichia coli* and total bacteria specific primers sets, respectively. The purified PCR products were then cloned into the PMD19-T vector (TaKaRa Biotechnology, Dalian, China) respectively and transformed into the competent cells of DH5α. One of the positive clones of each PCR product was cultured, and then the recombined DNA of the plasmid of each PCR product in the cultured positive clone was extracted by TIANprep Mini Plasmid Kit (Tiangen, Beijing, China) and used as the qPCR DNA standards. The concentration of these DNA standards was diluted to 30 ng/μL and was then subject to a serial of 10-fold dilutions to perform the calibration curves (Patra and Yu, 2014). The copy number of the diluted DNA standards was calculated based on the mass concentration and the average molecular weight of the recombined DNA following the equation described by Yu *et al.* (2005). The standard curve was prepared with log copies and Ct. The number of *Bacteroides-Prevotella, Escherichia coli* and total bacteria in each sample with 30 ng metagenomic DNA could be calculated from the calibration curves according to their Ct value and was used to deduce the number of *Bacteroides-Prevotella, Escherichia coli* and total bacteria in one gram of digesta by multiplying the original volume of the extracted DNA and its dilution multiple.

The reaction mixture (20 μL) of real-time PCR consisted of 10 μL of SYBR Premix Ex Taq (Takara Biotechnology [Dalian] Co., Ltd, Dalian, Liaoning, China), 0.3 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., Hercules, CA, USA) with an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45 s. The primers designed according to the sequence of bacterial 16s rDNA and used for detecting bacterial number are listed in Table 2 (Huijsdens *et al.,* 2002; Denman and Mcsweeney, 2006; Reagan *et al.,* 2009).

Real-time PCR assay for determination of the relative expression of interleukin-6 and interleukin-10

Total RNA of the vermiform appendix was extracted by TRIzol® Reagent (Invitrogen by Life Technologies, NY, USA) according to the manual of TRIzol® Reagent. The cDNA of each sample was synthesised using PrimeScriptTM RT reagent Kit (Takara Biotechnology (Dalian) Co., Ltd, Dalian, Liaoning, China).

The reaction mixture (20 μL) of real-time PCR consisted of 10 μL of SYBR Premix Ex Taq (Takara Biotechnology [Dalian] Co., Ltd, Dalian, Liaoning, China), 0.4 μM of each primer and 50 ng of cDNA. Real-time PCR was performed on Bio-rad-IQ5 PCR System (BioRad, Laboratories Inc., Hercules, CA, USA) with an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 45 s. The gene encoding Glyceraldehyde 3-phosphate

Table 2: The specific primers for determining total bacteria, *Bacteroides* and *Escherichia coli.*

Items	Primer	Primer sequence(5'-3')	Amplicon size (bp)
Total bacteria	Forward	CGGCAACGAGCGCAACCC	130 (125-146)
	Reverse	CCATTGTAGCACGTGTGTAGCC	
Bacteroides–Prevotella	Forward	AACGCTAGCTACAGGCTT	272
	Reverse	CCAATGTGGGGGACCTTC	
Escherichia coli	Forward	GTTAATACCTTTGCTCATTGA	340
	Reverse	ACCAGGGTATCTAATCCTGTT	

Table 3: The specific primers for quantitative real-time polymerase chain reaction analysis of IL-6 and IL-10.

Items	Primer	Primer sequence(5'-3')	Amplicon size (bp)
GAPDH	Forward	TGACGACATCAAGAAGGTGGTG	120
	Reverse	GAAGGTGGAGGAGTGGGTGTC	
IL-6	Forward	CTACCGCTTTCCCCACTTCAG	135
	Reverse	TCCTCAGCTCCTTGATGGTCTC	
IL-10	Forward	CTTTGGCAGGGTGAAGACTTTC	126
	Reverse	AACTGGATCATCTCCGACAAGG	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL-6: interleukin 6; IL-10: interleukin 10.

dehydrogenase (GAPDH) was served as the housekeeping gene. The primers for investigating each gene are listed in Table 3 (Schnupf and Sansonetti, 2012).

Assay of mucosal sIgA in vermiform appendix

The concentration of mucosal sIgA in vermiform appendix was determined according to Wang (1998). Specifically, 0.3 g mucosa scraped from the middle part of vermiform appendix was dissolved in 1 mL of phosphate buffer that contained 2 μg/mL aprotinin and 2 μg/mL pepstatin. Samples were treated by ultrasonication for 20 min and then centrifuged at 12000×*g* for 30 min. Supernatants were collected to analyse the concentrations of sIgA, which were determined using the rabbit sIgA ELISA kit (YanJin Biological, Shanghai, China).

Investigation of intestinal morphology of suckling rabbits

The intestinal sections submerged in a fixative solution for 3 d were rinsed with water and then dehydrated in a graded series of absolute ethanol (50%, 70%, 80%, 90%, 100%) before clearing with benzene twice. The dehydrated sections were saturated with and embedded in paraffin. Sections of 6 μm thickness (10 slices of each sample) were stained with haematoxylin/eosin and prepared for observation by light microscopy. A total of 10 intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section. Villus height was measured from the tip of the villi to the villus crypt junction. Crypt depth was defined as the depth of the invagination between adjacent villi. The intestinal wall thickness of appendix was measured from the tip of the crypt to the epicuticle. The morphological measurements were performed using an image processing and analysis system (Optimus software version 6.5, Media Cybergenetics, North Reading, MA).

Statistical analysis

All data were analysed by Student's t-test (SPSS18.0). Significance was declared at $P<0.05$.

RESULTS

Effect of DLS on growth performance of suckling rabbits

As shown in Table 4, the average daily gain (g/d) at 3rd wk ($P=0.027$), 4th wk ($P=0.045$) and the whole stage ($P=0.003$) in DLT group were higher than that in DLS group. There was no difference in diarrhoea incidence between the 2 groups (data not shown), and no kits or does died in either group during the whole trial.

Table 4: Effect of DLS on average daily gain per week and the whole stage (mean±standard error).

Item	1st wk	2nd wk	3rd wk	4th wk	Whole stage
DLT	5.83±1.04	12.57±0.76	15.86±1.07	28.54±1.51	15.7±0.5
DLS	5.35±0.93	12.54±1.21	13.36±1.36	26.11±1.20	14.3±0.2
P-value	0.529	0.962	0.027	0.045	0.003

DLS: doe-litter separation; DLT: doe-litter together.

Effect of DLS on the number of Escherichia coli*,* Bacteroides-Prevotella *and total bacteria in caecum and duodenum content*

We investigated the number of bacteria in caecal and duodenal content by real-time PCR with bacteria-specific primers. The results are shown in Table 5. At 7 d of age, kits in DLS group had fewer caecal *Bacteroides-Prevotella* (P=0.047) and total bacteria (P=0.046), and fewer duodenal *Bacteroides-Prevotella* (P=0.024), *Escherichia coli* (P=0.042) and total bacteria (P=0.036). One week later, the number of caecal *Bacteroides-Prevotella* (P=0.001) and total bacteria (P=0.021), and duodenal *Escherichia coli* (P=0.033) in kits separated from their mother, were still remarkably lower than that in DLT group. At 21 d of age, the difference in number for the investigated bacteria between these 2 groups was not significant, expect for the number of *Escherichia coli* in duodenal content and total bacteria in caecal content. At the last day of the trial, caecal total bacteria in kits separated from their mother still tended to be lower than that in kits caged with their mother (P=0.067).

Effect of DLS on the relative expression of IL-6 and IL-10 in duodenum and vermiform appendix

The relative expression of IL-6 and IL-10 in duodenum and vermiform appendix was investigated by real-time PCR and the results are presented in Table 6. Expression of IL-6 in duodenum and vermiform appendix was not affected by doe-litter separation (P>0.05). Kits in DLS group expressed less IL-10 in these 2 intestinal segments from the onset to the end of trial. On day 14 and day 28, DLT had higher expression of IL-10 in duodenum and vermiform appendix (P=0.031 and P=0.043, respectively).

Effects of DLS on the secretion of sIgA in duodenum and vermiform appendix

We measured the concentration of mucosal sIgA in duodenum and vermiform appendix by ELISA. The results are shown in Table 7. The concentration of mucosal sIgA in duodenum was not affected by DLS throughout the trial (P>0.05), and kits in DLS group had lower concentration of mucosal sIgA in vermiform appendix only at 28 d of age (P=0.042).

Table 5: Effect of DLS on the number of bacteria in caecum and duodenum (mean±standard error).

Item			7th d	14th d	21st d	28th d
Bacteroides-Prevotella	Duodenum	DLT	4.13±0.44	5.19±0.62	6.16±0.66	7.74±0.86
(log copies/g content)*		DLS	3.21±0.21	5.06±0.35	5.89±0.71	7.33±0.70
		P-value	0.024	0.736	0.609	0.488
	Caecum	DLT	5.33±0.64	6.72±0.49	8.33±0.73	8.97±0.86
		DLS	4.38±0.42	5.53±0.51	7.56±0.99	8.82±0.71
		P-value	0.047	0.001	0.492	0.846
E. coli	Duodenum	DLT	2.11±0.16	3.04±0.19	4.16±0.44	3.95±0.16
(log copies/g content)		DLS	1.42±0.39	2.36±0.27	2.53±0.48	3.33±0.33
		P-value	0.042	0.033	0.007	0.128
	Caecum	DLT	1.02±0.12	2.90±0.39	4.95±0.29	6.13±0.49
		DLS	1.05±0.11	2.59±0.42	4.54±0.52	5.51±0.77
		P-value	0.741	0.321	0.171	0.219
Total Bacteria	Duodenum	DLT	6.07±0.63	7.00±0.43	7.52±0.99	8.47±0.95
(log copies/g content)		DLS	5.09±0.60	6.53±0.88	7.02±0.39	8.09±1.01
		P-value	0.036	0.367	0.381	0.641
	Caecum	DLT	6.81±0.38	8.08±0.76	9.62±0.31	10.13±0.57
		DLS	5.85±0.72	6.92±0.56	8.71±0.53	9.39±0.34
		P-value	0.046	0.021	0.025	0.067

DLS: doe-litter separation; DLT: doe-litter together.

*log copies/g content: log copy numbers of 16S rDNA/g content.

Table 6: Effect of DLS on the relative expression of IL-6 and IL-10 in duodenum and appendix (mean±standard error).

Item			7th d	14th d	21st d	28th d
IL-6	Duodenum	DLT	1.79±0.36	2.50±0.13	3.38±0.20	3.17±0.07
		DLS	1.61±0.26	2.30±0.26	3.30±0.19	3.11±0.14
		P-value	0.444	0.226	0.452	0.287
	Appendix	DLT	1.04±0.07	1.86±0.30	2.43±0.06	2.59±0.42
		DLS	1.24±0.31	1.73±0.33	2.49±0.11	2.46±0.18
		P-value	0.460	0.574	0.310	0.425
IL-10	Duodenum	DLT	1.63±0.24	1.88±0.29	2.88±0.43	3.04±0.40
		DLS	1.14±0.32	1.28±0.25	2.13±0.28	2.36±0.38
		P-value	0.042	0.031	0.058	0.064
	Appendix	DLT	3.59±0.53	6.44±0.66	13.87±0.87	26.06±1.20
		DLS	2.95±0.47	5.27±0.65	11.30±1.19	23.04±2.17
		P-value	0.089	0.051	0.101	0.043

IL-6: interleukin 6; IL-10: interleukin 10; DLS: doe-litter separation; DLT: doe-litter together.

Effect of DLS on intestinal morphology

As shown in Table 8, DLS had no effect on the kit's duodenal morphology before feeding solid food, but decreased the villus height: crypt depth ratio on day 21 (P=0.03) and day 28 (P=0.02) of age. Kits separated from their mother had lower villus height (P=0.025) and higher crypt depth (P=0.039) at the end of the trial. Both intestinal wall thickness and length of vermiform appendix were affected by doe-litter separation. Kits separated from their mother had thin intestinal wall thickness of vermiform appendix during the last two weeks (P=0.044 and 0.049) and shorter appendix at the end of the trial (P=0.029).

DISCUSSION

Development of the intestinal microbiota in mammals starts at birth and is influenced by many factors (Kirjavainen and Gibson, 1999; Penders *et al.,* 2006; Eiben *et al.,* 2007). As we supposed, the study presented here proved that doe-litter separation reduced the number of bacteria in both duodenum and caecum of suckling rabbits, especially before feeding creep feed. But the effect of doe-litter separation on duodenal microflora was not consistent with that on caecal. The number of *E. coli* in duodenal content was significantly decreased by DLS from beginning to the third week, while that in caecal content was not affected throughout the trial. Kits separated from their mother had fewer total bacteria in caecal content from the beginning to the end. This difference is probably due to the pH difference between duodenal and caecal content. Rabbit caecum has lower pH, and therefore the number of caecal *E. coli* is not likely to be changed.

Analyses indicated that profound changes occur in the intestinal ecosystem when young mammals are weaned from their mother's milk onto solid food (Mackie *et al.,* 1999). Our study showed that the influence of doe-litter separation

Table 7: Effect of DLS on secretion of sIgA in duodenum and vermiform appendix (μg/100 mg mucosa (mean±standard error).

Item		7th d	14th d	21st d	28th d
Duodenum	DLT	0.41±0.05	0.60±0.06	1.28±0.12	2.48±0.25
	DLS	0.39±0.06	0.62±0.11	1.18±0.10	2.39±0.26
	P-value	0.586	0.753	0.245	0.636
Appendix	DLT	0.64±0.08	1.58±0.21	2.55±0.26	5.16±0.47
	DLS	0.62±0.08	1.55±0.35	2.37±0.23	4.23±0.55
	P-value	0.702	0.896	0.351	0.042

DLS: doe-litter separation; DLT: doe-litter together.

Table 8: Effect of DLS on intestinal morphology and length of appendix (mean±standard error).

Item			7th d	14th d	21st d	28th d
Duodenum	villus height (μm)	DLT	121.90±3.53	182.97±4.01	232.20±14.14	295.96±9.29
		DLS	119.96±5.00	181.06±3.86	227.25±8.63	278.51±7.17
		P-value	0.562	0.5191	0.572	0.025
	crypt depth (μm)	DLT	28.13±1.67	34.82±1.44	38.72±0.77	44.87±1.06
		DLS	28.82±0.97	35.18±1.24	39.61±0.61	48.02±1.71
		P-value	0.505	0.739	0.122	0.039
	V/C	DLT	4.24±0.10	5.22±0.09	6.14±0.05	6.65±0.07
	DLS	4.16±0.05	5.20±0.15	5.80±0.08	5.85±0.17	
	P-value	0.265	0.880	0.003	0.002	
Appendix	crypt depth (μm)	DLT	96.19±0.86	149.87±10.02	200.05±8.06	266.01±26.99
	DLS	95.95±1.08	148.87±9.98	198.55±6.85	265.59±33.81	
	P-value	0.724	0.902	0.725	0.983	
	IWT (μm)	DLT	203.03±9.62	332.79±14.40	445.98±16.74	644.39±22.60
	DLS	205.02±16.80	331.79±13.42	421.60±15.48	614.78±20.47	
	P-value	0.845	0.923	0.044	0.049	
	appendix length (cm)	DLT	2.74±0.10	3.58±0.22	4.58±0.11	5.75±0.19
	DLS	2.70±0.19	3.48±0.21	4.45±0.11	5.44±0.13	
	P-value	0.784	0.622	0.230	0.029	

DLS: doe-litter separation; DLT: doe-litter together.
V/C: villus height/crypt depth; IWT: intestinal wall thickness.

on the number of the investigated bacteria was obvious during the first 2 wk, especially the 1st wk, but became weak after kits were provided with supplementary food. This suggested that solid food is the main factor that affects the intestinal microbiota after kits being supplied with solid food.

There is increasing evidence to prove that microbiota, especially some commensal bacteria in the gastrointestinal tract, exert profound effects on the functional development of the intestinal tract, such as stimulating immunological and gut morphological development (Thompson *et al.,* 2008; Conroy *et al.,* 2009). *Bacteroides* is believed to be the major commensal genus in the gut tract of rabbit and *Bacteroides fragilis* had been proven to stimulate the development of gut associated immune tissue when injected to rabbit's vermiform appendix combined with *B. subtilis* or *S. epidermidis* (Rhee *et al.,* 2004; Hanson and Lanning, 2008). In the present study, kits separated from doe had fewer caecal *Bacteroides-Prevotella* for the first 2 wk, but this reduction did not affect intestinal morphology and immune responses synchronously. While during the last 2 wk, when there were no statistical difference in the number of caecal *Bacteroides-Prevotella* between the 2 groups, kits separated from their mother had less secreted sIgA and expressed interleukin-10 (known as an anti-inflammatory cytokine) in vermiform appendix and worse morphological parameters in duodenum. It has been reported that commensal bacteria stimulated follicle development and intrafollicular B cell proliferation in rabbit (Hanson and Lanning, 2008). More intrafollicular B cell in DLT kits may contribute to the higher intestinal sIgA. The previous higher number of caecal *Bacteroides-Prevotella* in DLT group probably had a subsequent positive effect on the development of intestinal immune tissue, or some other commensal bacteria not investigated might be higher in intestine of DLT kits and stimulated the development of intestinal tissue synchronously, and therefore kits caged longer with their mother had favourable gut immunological and morphological development. The increased intestinal sIgA and favourable state of inflammatory level may strengthen rabbit's resistance to disease after weaning. The stimulatory effect of commensal bacteria on the development of intestinal immune system was proved by the increased length and intestinal wall thickness of vermiform appendix.

The fact that the increased total bacteria in caecum and *E. coli* in duodenum of kits caged with their mother did not lead to higher interleukin 6 (a pro-inflammatory cytokine) or higher diarrhoea and morality rate suggested that the increase of intestinal bacteria in kits caged with their mother during suckling time had no negative effect on their gut

health. Our data showed the absolute number of intestinal *E. coli* of healthy kits increased with age, but this increase did not lead to gut disease. We also analysed the relative rate of *E. coli* to total bacteria in intestine of health rabbit (data not shown), and we found that it kept relatively stable with age. This led us to believe that, compared with the absolute number of intestinal pathogenic bacteria, the relative rate of pathogenic bacteria to total bacteria is a more accurate index of gut infection.

Doe-litter separation decelerated the growth of suckling rabbits after feeding solid food. And the difference in growth performance between the 2 groups was consistent with the difference in morphological parameters and sIgA concentration of intestine between the 2 groups. The positive effect of doe-litter together on the development of digestive or/and immune tissue contributed to the better growth performance. This consistence may lead us to suspect that rabbits caged longer with their mother might suckle more milk after supplementary feeding than kits in DLS group, and thus got better nutrition. But it is known that the doe naturally nurses kits only once or twice each day, and the nursing time lasts only about 5 min each time (http://fohn.net/rabbit-pictures-facts/rabbit-diet-digestive-tract-reproduction.html). To avoid the probable difference in nursing time between the 2 groups, we caged the kits with their mother twice each day in the DLS group.

Combes *et al.* (2011) reported that the number of *Bacteroides-Prevotella* in rabbit's intestine increased with increasing age from day 14 to day 21, and remained stable from 21 to 35 d of age and then fell. But the present study showed that the number of *Bacteroides-Prevotella* in kit's gut increased persistently with increasing age before weaning.

CONCLUSIONS

Compared with control, doe-litter separation decreased the number of the investigated intestinal bacteria, especially before feeding creep feed, and decelerated kit's growth, expression of interleukin, secretion of immunoglobulin and the development of vermiform appendix. Providing kits with material from the doe (such as soft faeces) may be a good strategy to avoid this deceleration when separating kits from the doe during the suckling period.

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