Original Article
Diversity and dynamic changes of intestinal microbial community in preterm infants with necrotizing enterocolitis

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Abstract: This study aimed evaluate the diversity and dynamics change of intestinal microbial community in preterm infants with necrotizing enterocolitis (NEC). Forty-two preterm infants were selected for fecal sample analysis, including 21 preterm infants with NEC and 21 healthy preterm infants. The diversity and dynamic changes of intestinal microbial community were explored through 16S rDNA PCR-denaturing gradient gel electrophoresis (DGGE). TA cloning Kit and sequencing were used to assess bacterial distribution and identify common dominant bacteria in preterm infants with NEC. Shannon index values for NEC stages I, II, and III were 2.01 ± 0.73, 1.93 ± 0.27, and 1.54 ± 0.36, respectively; the marched control groups CI, CII, and CIII showed 2.65 ± 0.26, 2.74 ± 0.16, and 2.56 ± 0.22, respectively. Shannon index of intestinal bacteria in the NEC group was significantly lower than that of the control group (P<0.05); meanwhile, Shannon index of NEC stage III was significantly lower than values obtained for NEC stages I and II (P<0.05). After treatment and gradual recovery, Shannon index of the NEC group was gradually increased and almost reached control values. There was no significant difference in the main bacterial types between NEC and control groups based on 16S rDNA PCR-DGGE, but *Bifidobacterium*, *Lactobacillus* and *Escherichiacoli* proportions in the NEC group were lower compared with control values. In contrast, *Klebsiella* and *Bacteroides spp.* Proportions were higher than those of the control group. The types and amounts of intestinal bacteria (especially probiotics) were reduced, but *Klebsiella* levels were increased, which might be an important factor in the pathogenesis of NEC.

Keywords: Necrotizing enterocolitis (NEC), microbiota, *Klebsiella*, probiotics

Introduction

Neonatal necrotizing enterocolitis (NEC) is considered the most common and serious gastrointestinal emergency in neonatal infants with amorbidity of 1-3‰; 90% of NEC occurred in preterm infants [1].

It is widely accepted that NEC is associated with multiple factors, including abnormal bacterial colonization [2]. However, it is difficult or impossible to culture 60-80% of gastrointestinal bacteria due to the limitations of traditional culture methods; consequently, culture results could not reflect the diversity of intestinal microbial community [3]. With the development of molecular biology techniques, such as 16S rDNA PCR-DGGE, microbial communities could be detected fully and accurately [4].

Previous studies have reported important differences in the gut microbiota of preterm infants with NEC [5, 6]. However, the relationship between change of intestinal microbial community and NEC remains largely undefined. This study aimed to explore the diversity of intestinal microbial community in preterm infants with NEC by 16S rDNA PCR-DGGE, TA Cloning Kit, and sequencing, focusing on the relationship between NEC severity and the change of intes-

Table 1. Clinical data of the NEC and control groups

Characteristics	Control (n=21)	NEC (n=21)	t/χ^2	P
Sex (male/female)	13/8	14/7	χ^2=0.101	0.286
Gestational age (d, $\overline{x} \pm s$)	242.2 ± 12.9	241.5 ± 11.7	t=1.369	0.186
Weight (kg, $\overline{x} \pm s$)	2.27 ± 0.51	2.25 ± 0.55	t=-0.244	0.810
Days of collecting samples (d, $\overline{x} \pm s$)	9.67 ± 6.13	9.76 ± 7.13	t=0.271	0.789
Birth methods (natural births/caesarean)	10/11	8/13	χ^2=0.380	0.648
Feeding methods (human milk/formula milk)	8/13	6/15	χ^2=0.418	0.167
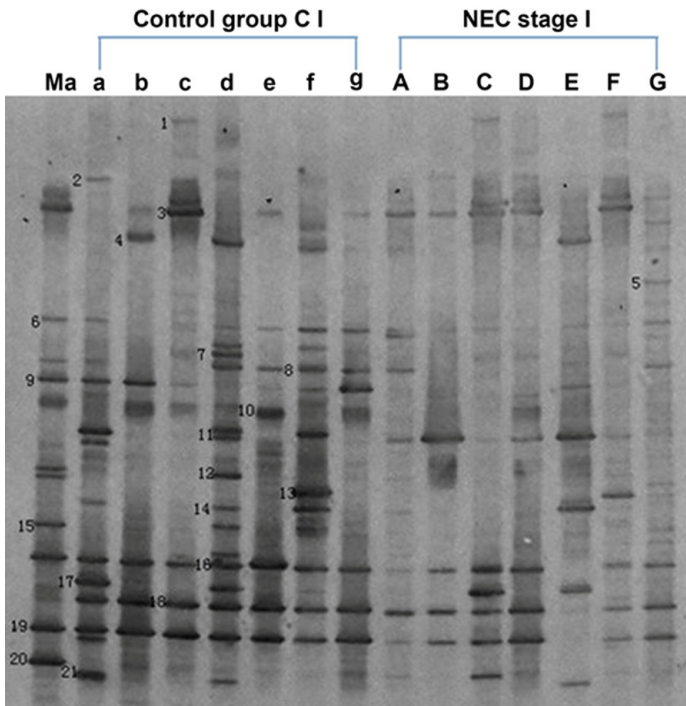
Figure 1. DNA 16S rDNA PCR DGGE of NEC stage I and control groups within 24 hours of admission. Notes Ma, Marker; NEC stage I (cases A-G) and control group I (casesa-g). The numbered bands were cut and sequenced.

tinal microbial community. Our findings provide a basis for understanding NEC mechanisms and could help improve its treatment.

Patients and methods

Between August 2009 and December 2012, preterm infants (<37 weeks of gestation) were recruited from the level III neonatal intensive care units (NICUs) of Shenzhen Children's Hospital and Children's Hospital of Chongqing Medical University, respectively. These hospitals are 2 of the busiest newborn tertiary centers in China. Twenty-one preterm infants with NEC were included based on Bell NEC criteria,

and those with congenital intestinal diseases were excluded. The enrolled preterm infants were divided into NEC stages I (n=7), II (n=7), and III (n=7), respectively, based on Bell clinical staging [7]. Fecal samples of the infants were collected during the first day of hospitalization (21 samples). Due to fasting, fecal samples from only 10 preterm infants were collected at 1, 3, 5, 7, and 9 days upon admission. Fecal samples were assessed for dynamic changes of the intestinal microbial community. Meanwhile, a control group of preterm infants matched with the NEC group in terms of C staging (CI, CII, and CIII), basic diseases, gestational age and weight was constituted (**Table 1**). All infants underwent formula-feeding and administered broad spectrum antibiotics or second generation cephalosporins.

Ethics statement

This study was approved by the Children's Hospital of Chongqing Medical University, Chongqing, China, and performed in accordance with the Declaration of Helsinki. Written informed consent for postoperative collection of samples was provided by each infant's parents after consultation.

Sample preparation and storage

Fecal samples were stored at -80°C until analysis. Bacterial DNA was extracted from 0.22 g of fecal samples using QIAamp Stool MiniKit (QIAGE N, German), in accordance with the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification PCR amplification of bacterial DNA was carried
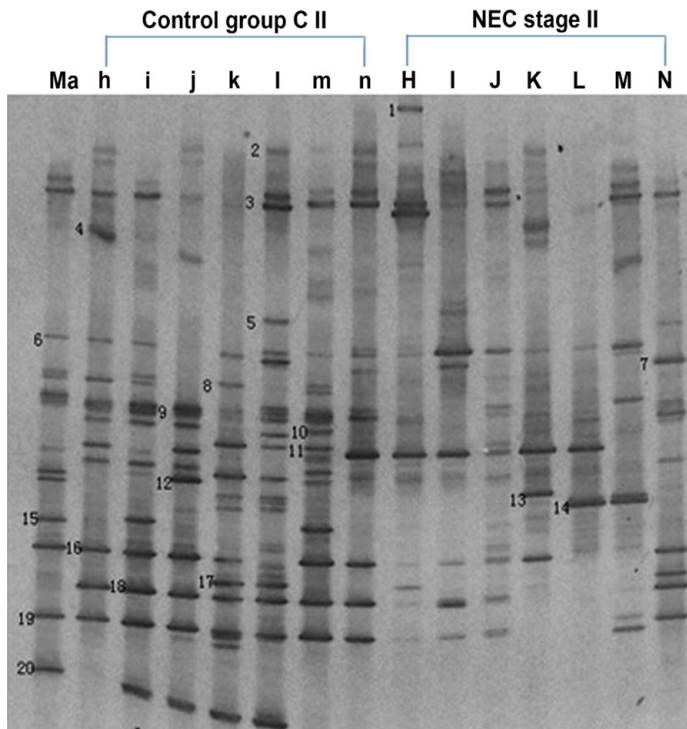
Figure 2. DNA 16S rDNA PCR DGGE of NEC stage II and control groups within 24 hours of admission. Notes Ma, Marker; NEC stage II (cases H-N) and control group II (casesh-n). The numbered bands were cut and sequenced.
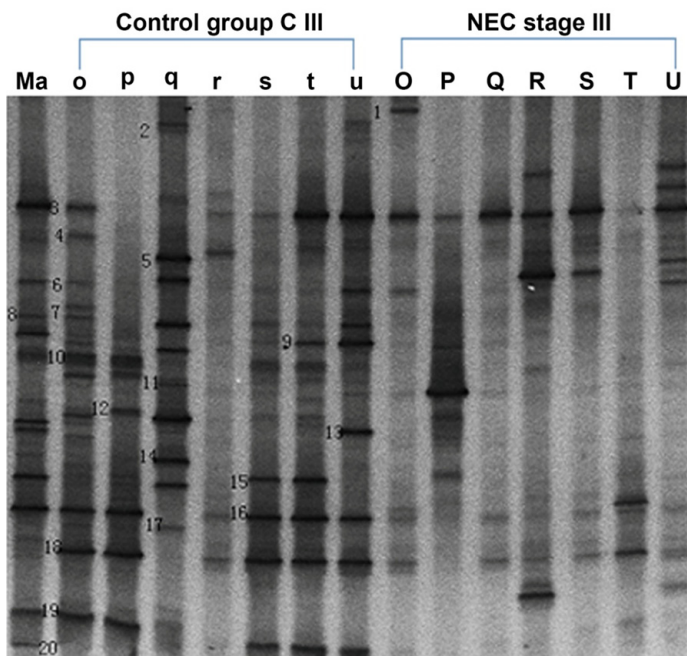
Figure 3. DNA 16S rDNA PCR DGGE result of NEC stage III and control groups within 24 hours of admission. Notes Ma, Marker; NEC stage III (cases O-U) and control group III (caseso-u). The numbered bands were cut and sequenced.

out with the following primer sets for 16S rDNA V3: 5'-CGCCCGGGGC-GCGCCCCGGGCGGGGCGGGGGCAG-GGGCCTACG-GGAGGCAGCAG-3' and 5'-ATTACCGCGGCTGCTGG-3' (Invitrogen). 50 μl PCR reactions included 1 μL primers (10 μmol/L), 5 μL DNA-template, 25 μL premix Taq (Takara Biotechnology Co.Ltd, Dalian), and 19 μl deionized water. PCR conditions were: 94°C for 2 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; 72°C for 5 min. PCR products were detected by 2% agarose electrophoresis [8, 9].

Denaturing gradient gel electrophoresis and sequencing

Based on our previous methods for DGGE, 8% polyacrylamide and 35-65% gradient gels were used for DGGE analysis on a Dcode™ general mutation detection system (BIO-RAD, USA). A total of 20 μl PCR product was added to 1×TAE electrophoresis buffer, and submitted to electrophoresis (85 V, 60°C) for 16 h. Staining was carried out with SYBR green I (BioTek) for 30 min, and gel analysis performed on a Bench-Top3 UV transilluminator. Target DNA was recycled and amplified as described previously [8, 9].

TA cloning and sequencing

PCR products were purified and recycled using a TaKaRa kit, and submitted to TA cloning (TAKARA pMD-18T Vector), sequencing and analysis. Results were compared with the NCBI GenBanknucleotide database (http://www.ncbi.nlm.nih.gov) using BLAST.

16S rDNA PCR-DGGE image analysis

16S rDNA PCR-DGGE images were analyzed using the Quantity One software. Band number represented the species richness (S) of the intestinal microbial community. Shannon Wiener Index (H') was calculated

Table 2. Band richness and Shannon index of the NEC and control groups within 24 hours of admission

	Group		Group		Group	
	NEC stage I (n=7)	CI (n=7)	NEC stage II (n=7)	CII (n=7)	NEC stage III (n=7)	CIII (n=7)
Band richness	7.57 ± 0.53*	14.71 ± 3.82	7.14 ± 1.77*	15.86 ± 2.61	5.00 ± 1.73*,#	13.29 ± 2.75
Shannon index	2.01 ± 0.73*	2.65 ± 0.26	1.93 ± 0.27*	2.74 ± 0.16	1.54 ± 0.36*,#	2.56 ± 0.22

Notes *: Diversity was lower inNEC stages I, II, and III compared with the control group (P<0.05). #, Compared with NEC stages I and II, NEC stage III showed lower diversity (*P*<0.05). NEC stages I and II showed similar diversity (*P*>0.05). No statistically significant difference in was obtained among control subgroups CI, CII and CIII (*P*>0.05).
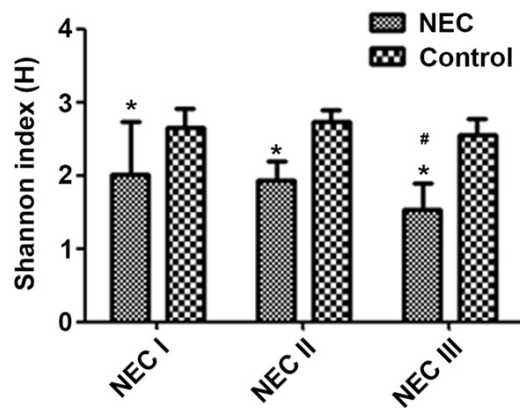
Figure 4. Shannon index in the NEC and control groups within 24 hours after admission. Notes *: Compared with the control group, diversity in NEC stages I, II, and III was lower (P<0.05); #: Compared with NEC stages I and II, diversity in NEC stage III was lower (*P*<0.05). Similar diversity was found in NEC stages I and II (*P*>0.05). No statistically significant difference was found in diversity among the control subgroups CI, CII and CIII (*P*>0.05). (Comparisons between two groups were performed using the independent samples *t*-test, and comparisons between many groups were performed using one-way ANOVA).

based on the Biodiversity Data Analysis Package Biadapsoftware, reflecting a positive correlation with evenness extent of band number (bacteria types) and optical density (bacteria number).

Statistical analysis

All statistical analyses were performed with SPSS version 19 (SPSS, USA). Count data were used in the χ^2 test when n≥40 and T≥1, and the extract method was used when n<40 or T<1. The data with a normal distribution were expressed as *x* ± *s*. Comparisons between two groups were performed using the independent samples *t*-test, and comparisons between many groups were performed using one-way ANOVA. Two sided P<0.05 was considered statistically significant.

Results

Microbial diversity at 1 day of hospitalization

Fecal samples from the NEC (n=21) and control groups (n=21) during the first day of hospitalization were analyzed on gradient gels (16S rDNA PCR DGGE). **Figures 1-3** are representative data for stages 1, 2 and 3 NEC. Comparisons between two groups were performed using the independent samples *t*-test, and comparisons between many groups were performed using one-way ANOVA. As shown in **Table 2**, the diversity of intestinal microbial community in the NEC group was lower than that of the control group; in addition, the diversity of intestinal microbial community was lower in NEC stage III than in NEC stages I and II (**Figure 4**).

Dynamic changes of intestinal microbial community

A total of 20 fecal samples from 10 NEC and 10 control patients were obtained at different time points after admission. A total of 5, 3, and 2 fecal samples were obtained for NEC stage I, II, and III patients, respectively. The corresponding DNA bands for the NEC and control groups were detected by DGGE (**Figure 5**). DNA bands for the NEC group gradually lost intensity in convalescence. These results indicated that after treatment and gradual recovery, the diversity of intestinal microbial community in NEC patients was increased gradually, almost reaching that of the control group at 9 days (*P*>0.05); the diversity of intestinal microbial community in the control group was steady throughout the study (**Figure 6**).
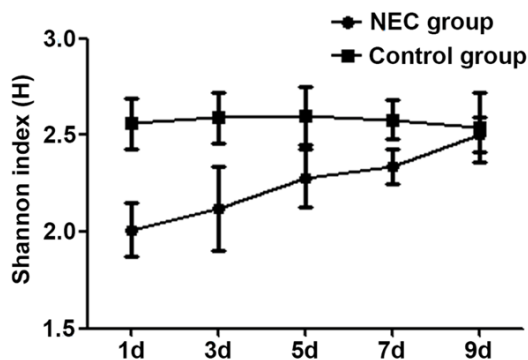
Bacterial distribution at 1 day of hospitalization

Predominant bands from DGGE data (marked 1-21) were recycled, sequenced, and comparatively analyzed (Http://blast. ncbi.nlm.nih.gov/
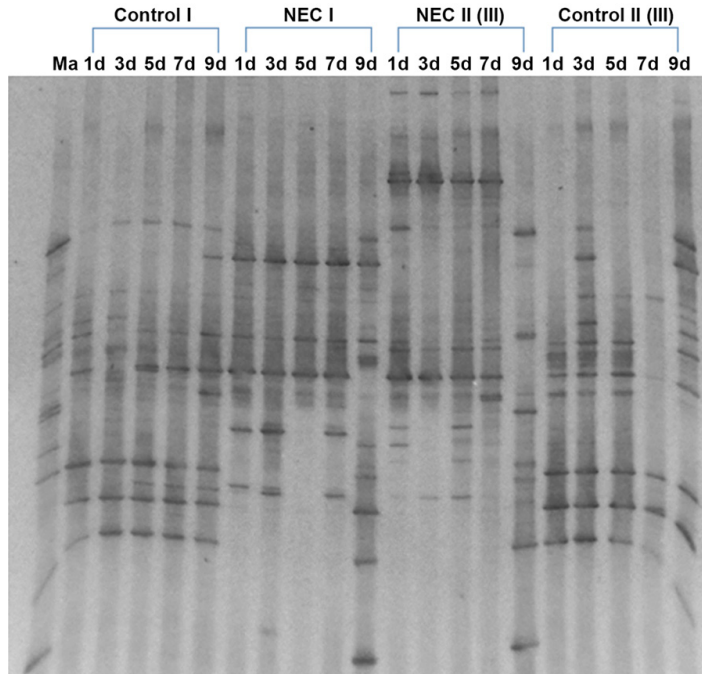
Figure 5. Fecal 16S rDNA PCR DGGE results of the NEC and control groups at different time points after admission.

Figure 6. Dynamics analysis of DNA Shannon index between NEC group and control group. Notes *: Compared with the control group, the NEC group showed lower DNA Shannon index values at 1, 3, 5, and 7 days after admission (P<0.05). 9 days after admission, DNA Shannon index in the NEC group almost approached to the control value (P>0.05).

Blast). As shown in **Table 3**, all bacteria were divided into 11 kinds, and their proportions in the control and NEC groups are shown in **Figure 7**. No significant differences in bacterial types were found between the control and NEC groups. The proportions of *Bifidobacterium*, *Lactobacillus* and *Escherichia coli* were lower, while those *Klebsiella* species and *Bacteroides* were increased in the NEC group compared with controls.

Discussion

It is important to maintain the balance of host cells, immune cells and intestinal bacteria; indeed, any abnormality in intestinal environment may lead to NEC [10]. Intestinal bacteria have many physiological functions, including nutrition adsorption, fat distribution, adjustment of the intestinal mucosal epithelial barrier, and regulation of congenital and acquired immunity. Abnormal colonization of intestinal bacteria may regulate one of the mechanisms that mediate NEC [2, 11, 12]. However, recent studies revealed that more than 60-80% of microbes in nature are difficult to culture in the laboratory using traditional culturing methods [13]. This study could identify more than 1% of predominant bacteria in the microbial community by 16S rDNA PCR DGGE with high sensitivity, acceptable cost, and practicality, fully reflecting the bacterial distribution.

The above results demonstrated that the diversity of intestinal microbial community in the NEC group was lower than that of the control group, with NEC stage III showing a lower level compared with NEC stages Iand II. After treatment and gradual recovery, the diversity of intestinal microbial community in the NEC group was gradually increased, almost reaching the control level at 9 days. Thus, we speculate that the types and quantity of intestinal bacteria (especially probiotics) were reduced because of various risk factors. The diversity of intestinal microbial community in NEC is disputable. Some studies found no difference in diversity between preterm infants with NEC and controls [14-16]. Meanwhile, others reported that intestinal bacterial diversity is reduced compared with the control group, and considered the abnormal proportion of intestinal bacteria an important risk factor, in agreement with the current study [6, 17, 18]. Furthermore, Valarie E McMurtry [19] demonstrated that low bacterial diversity in stool specimens may be indicative of presence and severity of NEC, also corroborating our findings.

Probioticsare mainly located in the ileumand beneficial to the diversity and growth of the

Table 3. Cloning and sequencing data from DGGE

Band number	Bacteria	Sequence number	Similarity	Frequency of occurrence Control group/ NEC group	No. Control group	No. NEC group
1	Bacteroides	JQ680134.1	100%	1/4	C	CFHO
2	Bifidobacterium	KC160496.1	100%	11/3	Acdfhjlmnqu	BHK
3	Enterococcus faecalis	KF745071.1	100%	16/15	Bceghijlmnoqrstu	ABCDFHJMNOPQRSU
4	Enterococcus durans	KF250872.1	100%	13/2	Bdfhijmoqrstu	EM
5	Bacteria couldnot be cultured	GQ996402.1	99%	3/4	Lqr	GINU
6	Lactobacillus	AB889713.1	100%	16/6	Adefghiklmnoqstu	AIJMRS
7	Streptococcus	KF561353.1	100%	4/4	Dlno	INRS
8	Streptococcus salivarius	KF303402.1	100%	12/4	Defghikmqstu	AGKM
9	Klebsiella	KF835726.1	100%	15/7	Abcfghijlmnqstu	EHJKLNP
10	Klebsiella couldnot be cultured	KF680978.1	100%	16/5	Bcdefghijlmopstu	DKLRU
11	Escherichia coli	KF828880.1	99%	14/9	Adfhijklmnopqu	ABDEHIKLP
12	Bacillus could not be cultured	FR847072.1	95%	10/7	Djklmnopqt	BHIJKNP
13	Bacteria could not be cultured	JQ471644.1	98%	4/2	Fklu	FK
14	Arcobacter could not be cultured	FR837615.1	100%	6/4	Adfklq	ELMP
15	Escherichia Coli	KF418616.1	100%	7/1	Dfimqst	P
16	Klebsiella pneumoniae	KF551984.1	99%	20/15	Abcdefghijklmnoprstu	BCDFGIJKNOQRSTU
17	Klebsiella pneumoniae could not be cultured	JF815060.1	98%	5/4	Adklq	CEHN
18	Klebsiella	KF828865.1	100%	20/16	Abcdefghijklmnoprstu	ABCDFGHIJNOQRSTU
19	Escherichia Coli	KC768803.1	100%	20/11	Abcdefghijklmnopqstu	BCDFGIJMNRU
20	Rothia	KC632201.1	100%	5/2	Ijkl	CD
21	Bacteria could not be cultured	JQ998774.1	100%	2/1	AB	E
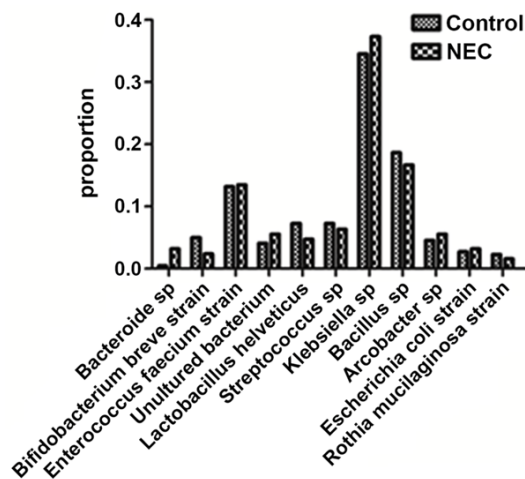
Figure 7. Proportions of individual bacterial species in the NEC and control groups.

intestinal microbial community, preventing the overgrowth of potential pathogenic bacteria [20, 21]. Probioticsinneonatal infants mainly include *Bifidobacterium* and *Lactobacillus*. Intestinal probiotic products were found to effectively reduce NECmorbidityas well as severity and mortality [22-24]. Although the detailed mechanisms mediating the effects of probiot-

ics on NEC remain unclear, the current study demonstrated that *Bifidobacterium* and *Lactobacillus* proportions in the NEC group were lower than control values, suggesting that a reduction in probiotics may lead to NEC, paving the way for prevention and clinical treatment of NEC.

Klebsiella pneumoniae is one of the normal intestinal bacteria but can cause diseases with changes of the intestinal environment. LEE et al found that *Klebsiella pneumoniae* increases the risk of colitis in a rat model [25]. Studies also found that Gram negative bacteria are associated with necrotizing enterocolitis [26, 27]. The current study likewise demonstrated that *Klebsiella pneumoniae* amounts in the NEC group were higher than control values, indicating this organism might be an important factor in the pathogenesis of NEC.

Collectively, types and quantity of intestinal bacteria (especially probiotics) were reduced by various risk factors, and *Klebsiella* was relatively predominant as an important factor causing NEC. Moreover, changes in the diversity of intestinal bacteria may be related to the severity of NEC. However, 16S rDNA PCR DGGE is as

emi-quantitative method, and quantitative real-time PCR (qRT-PCR) is needed to accurately estimate the differences of bacteria composition, exploring the effects of types and amounts of intestinal bacteria on NEC occurrence.

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Disclosure of conflict of interest

None.

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