

Bifidobacterium bifidum TMC3115 ameliorates milk protein allergy in by affecting gut microbiota: A randomized double-blind control trial

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Abstract

We aimed to explore the effects of *Bifidobacterium bifidum* TMC3115 intervention on cow's milk protein allergy (CMPA) and gut microbiota in infants. A total 256 CMPA infants were randomly and evenly assigned into an intervention group (*B. bifidum*) and a control group (placebo). Allergic scores, anti-inflammatory responses, and secondary outcomes were measured. Fecal specimens were collected, and the gut microbiota were analyzed by using 16S rDNA sequencing. After 6-month *B. bifidum* intervention, *B. bifidum* TMC3115 consumption reduced allergic scores, and improved anti-inflammatory responses and secondary outcomes in CMPA infants. LEfSe analysis showed *Bifidobacterium*, *Lactobacillus*, *Turicibacter*, *Sutterella*, and *Parabacteroides* were the most predominant phylum in the intervention group, whereas the Firmicutes (*Anaerovibrio*, *Christensenellaceae*, *Oscillibacter*, *Bilophila*, *Dorea*, and *Roseburia*) were most dominant phyla in the control group. Serum IgE or IgG2 had a strong relationship with α -diversity scores of gut microbiota.

Practical applications

There is a high level of prevalence of milk allergy in infants, which is difficult to be treated. *Bifidobacterium bifidum* TMC3115 supplement reduced allergic scores, improved anti-inflammatory responses, and reduced serum level of IgE and increased the level of IgG2 in the infants. On the contrary, *B. bifidum* supplement increased the genus proportion of probiotics and reduced the proportion of pathogens. The improvement of gut microbiota will be beneficial in the prevention of milk allergy. *B. bifidum* TMC3115 reduces milk allergy in the infants by regulating gut microbiota, and should be developed a potential way in the prevention of infant milk allergy.

KEYWORDS

Bifidobacterium bifidum, cow milk protein allergy, gut microbiota, IgE, infant

1 | INTRODUCTION

Cow milk is usually considered for the infants if their mothers cannot provide enough breast milk, but many infants will develop

allergic disease after cow milk consumption. Abnormal or excessive immune responses are often caused by cow milk protein allergy (CMPA) (Aktas et al., 2017). Milk allergy has a 2%–3% prevalence in infants (Meyer, 2008). The infants with a family history

of atopy and food allergy are at risk of developing milk allergy. Prevention and intervention treatments should be taken aggressively to control the allergic diseases. How to prevent and cure milk allergy, and reduce the antigenicity of milk protein has become the present research hotspots.

B. bifidum was the first isolated as probiotics from infant intestine and could improve immune homeostasis (Lopez et al., 2011, 2012). Because it is generally nonpathogenic, *Bifidobacterium* may have the advantage to produce various anti-allergic metabolites. *B. bifidum* has been found to improve rhinoconjunctivitis-specific quality of life in the individuals with seasonal allergy (Dennis-Wall et al., 2017). However, the effects of *B. bifidum* on CMPA infants remains unknown and related molecular mechanisms are still unclear.

The composition of gut microbiota is closely associated with allergic risk and significantly different between allergy and nonallergic infants (Penders et al., 2007). In comparison with nonallergic infants, intestinal *Lactobacillus* and *Bifidobacterium* and *Lactobacillus* are disappeared, and *Enterobacteriaceae* and *Staphylococcus aureus* are colonized more in some sick infants. In addition, *B. bifidum* has been found to have many health promoting properties, including anticancer (Heydari et al., 2018), antidiabetic (Sharma et al., 2016), anti-lipid accumulation (Wang et al., 2018), anti-inflammation (Philippe et al., 2011; Wang et al., 2016), and affect the profile of gut microbiota (Wang et al., 2019) so on. *B. bifidum* may have beneficial effects on CMPA infants by effecting gut microbiota.

B. bifidum TMC3115 treatment exerted beneficial effects on the serum cholesterol metabolism in the dyslipidemia patients by altering intestinal microbiota (Ke Wang et al., 2019). Oral administration of the strains TMC3115 could alleviate IgE-mediated allergies in adult host animals by regulating gut microbiota, SCFAs metabolism and anti-inflammatory cytokine IL-10 (Cheng et al., 2018). TMC3115 modulates intestinal microbiota and SCFAs metabolism, and affects gut microbiota and immunity. The results may be associated with the improvement of IgE-mediated allergies and related immune responses (Cheng et al., 2019). Therefore, more studies on gut microbiota profiling in CMPA are necessary to understand the function of *B. bifidum* TMC3115 on CMPA infants.

2 | MATERIALS AND METHODS

2.1 | Study hypothesis

We hypothesized that *B. bifidum* TMC3115 consumption would improve the milk allergy compared with the group with placebo.

2.2 | Study objectives

The objectives were to explore the clinical effect of *B. bifidum* TMC3115 on the infants with CMPA and possible mechanisms for the functional role of the probiotics.

2.3 | Study design

The present study was a double blind, randomized controlled trial with two parallel groups and the allocation ratio of 1:1. The study design is summarized in Figure 1.

2.4 | Cell culture of *B. bifidum* TMC3115

B. bifidum TMC3115 was purchased from the databank of Japan (Cat. NO. AP018131, Yata, Mishima, Shizuoka, JAPAN) and cultured anaerobically in MRS medium (Oxoid, Ogdensburg, NY, USA) in a 100-liter fermenter with 70% of liquid at stirring rate 100 r/min, 37°C for 48 hr. The quality of final species was approved by Institute of Microbiology, Chinese Academy of Sciences (Beijing, China) according to the quality assurance criteria for probiotic bacteria (Tuomola et al., 2001).

2.5 | Participants and recruitment process

The trial register No. is ChiCTR1900026918 and registered on October 26, 2019. The study was conducted at a single center, Affiliated Hospital of Changchun University of Traditional Chinese Medicine. Before the present experiment, all procedures were approved by the human research committee of Affiliated Hospital of Changchun University of Traditional Chinese Medicine (Approval No. CCZYFYLL2018) and the research has been carried out in accordance with the World Medical Association Declaration of Helsinki. Cow milk was purchased from Inner Mongolia MengNiu Dairy Co., Ltd. (Hohhot, China). From January 2016 to March 2017, we recruited the CMPA infants who could not be exclusively breastfed at the Department of Pediatric of our hospital. Informed written consent was obtained from each infant's guardian. Ten allergists in a team referred the CMPA cases to two research assistants for the present study. All infants were blinded to allocated treatment following evaluations, and all procedures were performed in a blinded fashion whenever possible. The infants joined the group according to the ID number in sequentially numbered containers they received.

2.6 | Diagnosis of CMPA

CMPA severely affects multiple organs and retards the growth and development of infants. Mucocutaneous symptoms include skin itching, eczema, urticaria, and lip, tongue, facial, and pharyngeal edema. Digestive symptoms include nausea, vomiting, diarrhea, flatulence, and intestinal cramps. Respiratory symptoms include runny nose, sneeze, stuffy nose, cough, and wheezing. Whole-body allergic reaction includes skin erythema, systemic symptoms, abnormal crying, and growth retardation.

Diagnostic tests for suspected milk allergy in infants included: a history of allergy; skin prick test; food-specific IgE test, and oral

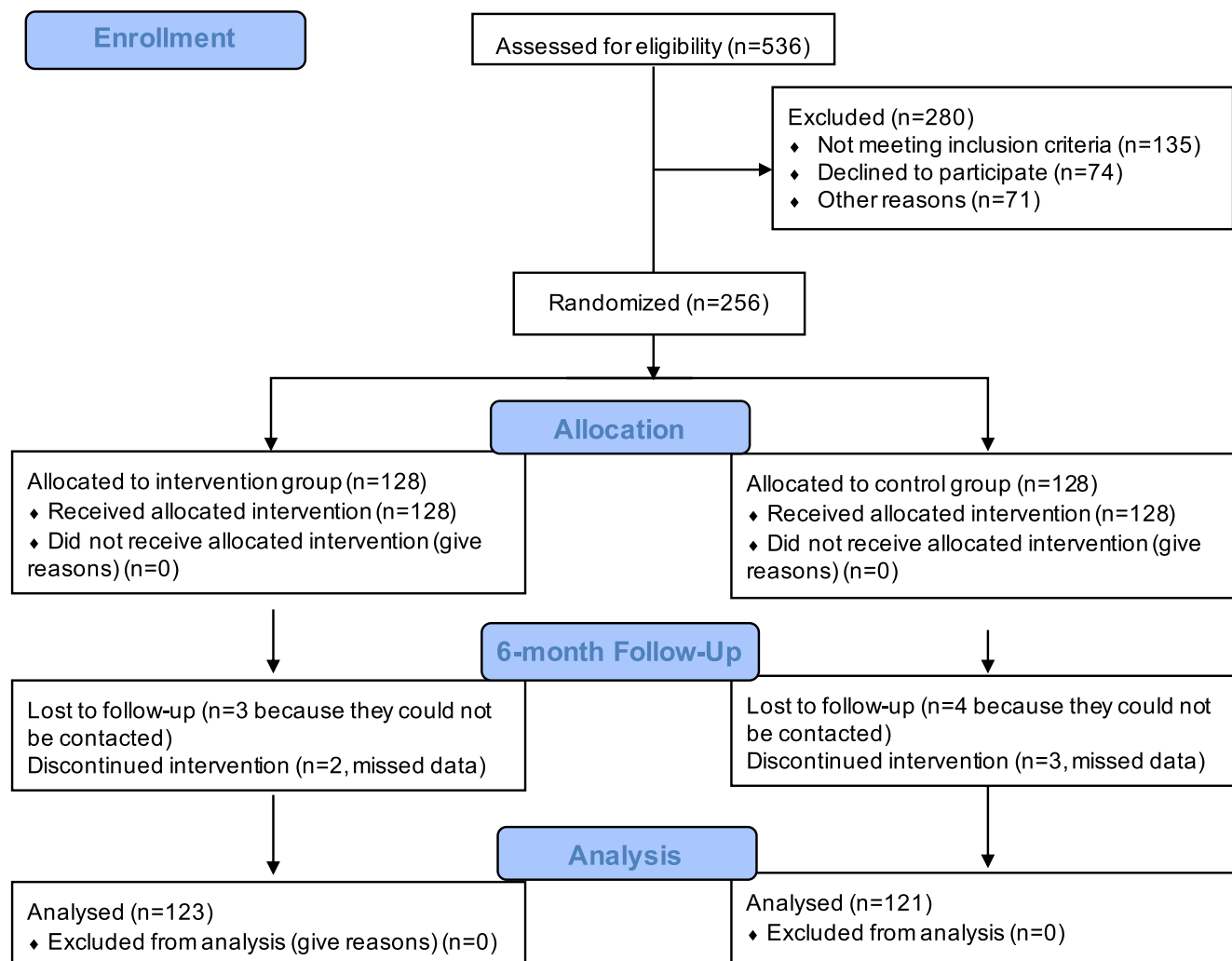


FIGURE 1 The Consolidated Standards for Reporting of Trials (CONSORT) of the present study diagram

food test. Double-blind and placebo-controlled food test was used in the diagnosis of milk allergy. Additionally, the currently developed labeling study for predicting milk allergy was particularly important for the early identification of infants with persistent milk allergies.

The following questions were considered for the infants with suspected CMPA: (a) the amount of intake of milk within a week; (b) the time for the occurrence of allergic symptoms after intake of cow milk; (c) the same allergic symptoms of the same milk; (d) the last time for the incidence of milk allergy; and (e) the presence of other risk factors.

Skin prick test (SPT) was used to measure IgE-mediated allergy disorders in the infants with rhino conjunctivitis, asthma, urticaria, anaphylaxis, atopic eczema, and milk allergy. Allergic severity (score) was evaluated by using eczema area and severity index score (Mochizuki et al., 2018). Histamine was used as the positive control and the saline was used the negative control. Positive results were determined as follows: food-induced specific eczema when compared with negative control; the diameters of eczema ranged from 3 to 4 mm, 5 to 8 mm, and >8 mm were marked as +, ++, and +++, respectively.

Two-milliliter peripheral venous blood was collected in an EDTA anticoagulant test tube. The blood was centrifuged at 2,000× g for

10 min at 4°C. Serum IgE was measured by using an immunoassay total serum IgE kit from Recipe Chemicals & Instruments GmbH (Munich, Germany). The serum levels of IgE were evaluated according to the quantitative concentration follows: <0.35 kU/L, 0; 35–0.70 kU/L, 1; 0.70–3.50 kU/L, 2; 3.50–17.50 kU/L, 3; 17.50–50.00 kU/L, 4; 50.00–100.00 kU/L, 5 and >100.00 kU/L, 6. While the serum levels of IgG2 were measured by using an IgG2 ELISA kit (Enzo Life Sciences Inc., Farmingdale, NY, USA).

All cases were treated with milk avoidance and positive provocation test by treating nondairy food suspected of being CMPA for 2 weeks. If the symptoms did not improve significantly, the infants were not necessarily allergic to milk. The infants would receive regular cow milk again if the symptoms were improved significantly. The infants would be diagnosed with milk protein allergy if the symptoms appeared again.

2.7 | Inclusion criteria

The infants had milk allergy after they consumed cow milk from Inner Mongolia Mengniu Dairy Co., Ltd. (Hohhot, China) and allergic

outcome was caused by the milk; the age was from 0.5 month to 12 months. They had the intermittent period of milk allergy of <half a month; they had >six times of milk allergy within 1 month and allergic symptoms duration of >half an hour at each time.

2.8 | Exclusion criteria

The following infants were excluded: the infants were allergic to *B. bifidum* TMC3115 and had serious organ failure, including renal, liver, lung, and heart disorders; and they underwent medical therapy for allergy; the infants are in a serious allergy situation (anaphylaxis). The allergy is caused by other reasons (such as bronchopulmonary dysplasia, primary ciliary dyskinesia, congenital respiratory tract deformity, mechanical obstruction, heart disease, neuromuscular abnormality, etc.) or primary immunodeficiency, and not by food milk. The infants had mental illness and could not cooperate to complete the experiment.

2.9 | Sample size calculation

A two-sample *t* test was used to calculate sample size by using GraphPad Prism (version 5.0, GraphPad Software Inc., La Jolla, CA, USA). Based on a minimally clinically important change (MCIC) of 10% on the allergy index for the skin prick test (Matricardi et al., 1994), standard deviation (S.D.) for the allergy index of 20% with significance level of $p < .05$, and power of 80%, at least 200 patients were needed for both groups at the primary endpoint. The current 256 patients allowed for a 20% loss to follow-up while keeping more than 80% power.

2.10 | Infants grouping

The random number was produced by a computer. Concealment of group assignment was performed for the all experts and researchers. As Figure 1 showed, there were 536 infants with allergic disease visiting Affiliated Hospital of Changchun University of Traditional Chinese Medicine (Changchun, China). After inclusion and exclusion selection, a total of 128 patients, who received oral administration of *B. bifidum* TMC3115 (2 ml of 1×10^9 cells/ml *B. bifidum* TMC3115 in 0.85% saline solution) at 7 a.m. each day at home for 6 months, comprised of the intervention group (BG). A total of 128 infants, who received 2 ml of 0.85% saline buffer at 7.0 a.m. each day for 6 months, comprised of the control group (CG). Breastfeeding shows protective role in infants with CMPA (Manti et al., 2017) and the duration of breastfeeding was compared between the two groups. Child's use of antibiotics has been found to be associated with an increased risk of cow's milk allergy (Metsala et al., 2013). Family history of allergy, mother's education, and pets at home have strong relationships with the risk of cow's milk allergy (Sardecka et al., 2018). All these parameters were also compared between the two groups.

2.11 | Selection bias

Selection bias was avoided by randomizing the allocated probiotic intervention. All infants were selected according to inclusion and exclusion criteria. Performance bias was avoided by using a standard process of medical care and schedule in our hospital between the both groups. Attrition bias was avoided as all randomized CMPA infants were included in the overall analysis.

2.12 | Evaluation of therapeutic efficacy

CMPA therapy was regarded as significantly effective and effective if skin eczema was reduced by more than 80% and 30%–80%, whereas the therapy was regarded as invalid if the skin eczema was reduced by less than 30%. The following parameters were investigated: nutrition intervention, milk allergy, the personal history, birth history, family history of allergy, feeding status, smoke exposure of mother, indoor environment, and clinical manifestations, etc. The patients were visited after 1-month and 6-month probiotics intervention.

2.13 | Follow-up study

Symptoms of an allergic reaction may involve the skin, the gastrointestinal tract, the respiratory tract, and whole-body allergic reactions. A nutrition file for each child was established to determine the feeding status, growth and development of the child before the visit, and the symptoms of milk protein allergy were scored according to allergic symptom index (Vandenplas et al., 2015). The allergic symptoms of infants were examined after *B. bifidum* treatment at 0, 1, and 6 months. Primary outcomes included the changes of allergic symptoms, and the serum levels of inflammatory cytokines after 1-month follow-up. Secondary outcomes included the changes of allergic symptoms, and serum level of IgE and IgG2, and the genus proportion of gut microbiota after 6 months of *B. bifidum* intervention.

2.14 | Assay of serum IgE and IgG2

Five-mL venous blood was obtained from each infant. Serum was prepared via the centrifugation at $2,000 \times g$ for 10 min at 4°C. Serum IgE and IgG2 were measured by using Human IgE (ab108650) ELISA Kit and Human IgG2 (ab202402) ELISA Kit (Abcam, Shrewsbury, MA, USA).

2.15 | Assay of serum inflammatory cytokines

The serum levels of human tumor necrosis factor α (TNF α) (MBS267654), human interleukin (IL)-1 β (#MBS175901), IL-6 (#MBS175877), and human IL-10 (#MBS175877) were detected by using the ELISA kits from MyBioSource, Inc. (San Diego, CA USA). The inflammatory factors were analyzed by using an

TABLE 1 Demographic characteristics between intervention group and placebo group

Parameters	<i>B. bifidum</i> group, <i>n</i> = 128	Placebo group, <i>n</i> = 128
Gender, male/female	72/56	76/52
Age, months	6.38 ± 5.52	6.24 ± 5.71
Age stages, months		
0–2, <i>n</i> (%)	20 (15.63)	22 (17.19)
3–4, <i>n</i> (%)	47 (36.72)	45 (35.16)
5–6, <i>n</i> (%)	29 (22.66)	26 (20.31)
7–8, <i>n</i> (%)	12 (9.38)	15 (11.72)
9–10, <i>n</i> (%)	10 (7.81)	12 (9.38)
11–12, <i>n</i> (%)	8 (6.25)	6 (4.69)
The time of birth		
Pre-term, <i>n</i> (%)	18 (14.06)	15 (11.72)
Full-term, <i>n</i> (%)	106 (82.81)	103 (80.47)
Post-term, <i>n</i> (%)	4 (3.13)	10 (7.81)
Breast-fed ≥3 months, <i>n</i> (%)	68 (53.1)	73 (57)
11 Used antibiotics, <i>n</i> (%)	45 (35.2)	39 (30.5)
Family history of allergy, <i>n</i> (%)	25 (19.5)	27 (21.1)
Pets at home, <i>n</i> (%)	11 (8.6)	13 (10.2)
Mother's education (≥college education), <i>n</i> (%)	65 (50.8)	60 (46.9)
Birthweight		
Low, <i>n</i> (%)	10 (7.81)	8 (6.25)
High, <i>n</i> (%)	95 (74.22)	98 (76.56)
Normal, <i>n</i> (%)	23 (17.97)	22 (17.19)
Body mass index, weight (kg)/height (m) ²	23.67 ± 3.16	22.98 ± 2.95
Allergic scores	9.28 ± 1.25	9.63 ± 1.06
Allergy grades		
0	6 (4.7)	3 (2.3)
1	35 (27.3)	38 (29.7)
2	47 (36.7)	45 (35.2)
3	22 (17.2)	27 (21.1)
4	14 (10.9)	12 (9.4)
5	3 (2.3)	2 (1.6)
6	1 (0.8)	1 (0.8)
Allergy symptoms		
Cough, wheeze	51 (39.84)	49 (38.28)
Runny nose, stuffy nose, sneezing	21 (16.41)	24 (18.75)
Nausea, vomiting, refusal to milk	25 (19.53)	26 (20.31)
Diarrhea, constipation	45 (35.16)	39 (30.47)

(Continues)

TABLE 1 (Continued)

Parameters	<i>B. bifidum</i> group, <i>n</i> = 128	Placebo group, <i>n</i> = 128
Bloating, intestinal cramps	13 (10.16)	17 (13.28)
Eczema (Urticarial and Swollen lips)	77 (60.16)	79 (61.72)
Itchy skin	23 (17.97)	20 (15.63)
Crying	15 (11.72)	17 (13.28)
Growth retardation	19 (14.84)	21 (16.41)

^aChi-square test.^b*t* test; the statistical difference was insignificant if *p* > .05.

automatic chemical analyzer (AU480, Beckman Coulter, Brea, CA, USA).

2.16 | Fecal microbiota analysis

About 5-mg fresh feces were obtained from individual infant before and after 1-, and 6-month *B. bifidum* intervention. Fecal DNA was isolated by using genome purification kit from TaKaRa (Dalian, China). The abundant genus in human gut microbiota was determined by qPCR in 96-well plates on a Real-Time PCR System (Bio-Rad, Hercules, Calif, USA). Fecal microbiota analysis was performed three replicates per sample. A 16S rRNA fragment was amplified using primers according to the previous report (Y. Wang & Qian, 2009). Each 20-μL qPCR mixture consisted of 10 μL of SYBR green PCR Master Mix (Roche, Indianapolis, IN, USA), 1.2 μL of a 100-nM of each primer, and 7.6 μL of genomic DNA template. The following PCR condition was used: 3 min at 95°C followed by 45 cycles of 30 s at 95°C, 20 s at 55°C, and 20 s at 72°C. The species were detected by using 16S rRNA sequencing on an Illumina HiSeq 2000/2500 platform (Dai et al., 2019). The sequences were grouped into an operational taxonomic unit (OTU) with 98% identity. The alpha_diversity was analyzed by using the number of OTUs, Chao1, Shannon diversity index, and Simpson diversity index (Casas et al., 2019). Beta diversity was evaluated by using weighted and unweighted UniFrac distances (Krishnamoorthy et al., 2020). The linear discriminant analysis (LDA) effect size (LEfSe) analyses were conducted on the website <http://huttenhower.sph.harvard.edu/galaxy>. The changes and relative abundance of genus proportion (mean values) of gut microbiota were plotted as heatmap and bar diagram.

2.17 | Pearson's correlation coefficient test between the levels of milk-specific IgE or IgG, and α-diversity scores of gut microbiota

Pearson's correlation coefficient test was used to explore the relationship between the levels of milk-specific IgE or IgG, and α-diversity

scores of gut microbiota (Chao1, observed species, PD_whole_tree, and Shannon diversity index).

2.18 | Statistical analysis

The changes of primary and secondary outcomes from baseline were analyzed using a two-tailed test with the significance level of 0.05. All data were presented as mean values $\pm$ SD. The missing data were not taken into account in the present study. All descriptive data were collected and constructed with 95% confidence intervals. Analysis of covariance (ANCOVA) was used to explore the differences in the mean values of the dependent variables. The normality was analyzed by using the Kolmogorov-Smirnov test. Continuous paired and categorical data were analyzed by using the Wilcoxon signed-rank test and a paired *t* test, respectively. The Mann-Whitney *U* test and unpaired *t* test were used to compare the clinical outcome between the two groups. Chi-square test were used to analyze the count data for baseline characteristics. Statistical analysis was conducted at recruitment, and after 1-month (primary outcome) and 6-month intervention (secondary outcome). All data were analyzed by using the software package

SPSS 20.0 (Statistics/IBM Corp, Chicago IL, USA). The statistical difference was considered significant for $p < .05$.

2.19 | Data management

All documents with specific ID were stored in locked filing cabinets and only accessible to designated researchers. The ID could not be identified by the researcher during the data analysis. Data record were carried out by a prearranged investigation helper. Any other data with infants' name were stored in different cabinets accessible by a nominated examiner.

3 | RESULTS

3.1 | Demographic characteristics

From January 2016 to March 2017, a total of 256 CMPA infants were selected in the present study. The original food intake habits and lifestyles were similar between two groups and remained unchanged. The statistical differences for breastfeeding duration, used antibiotics,

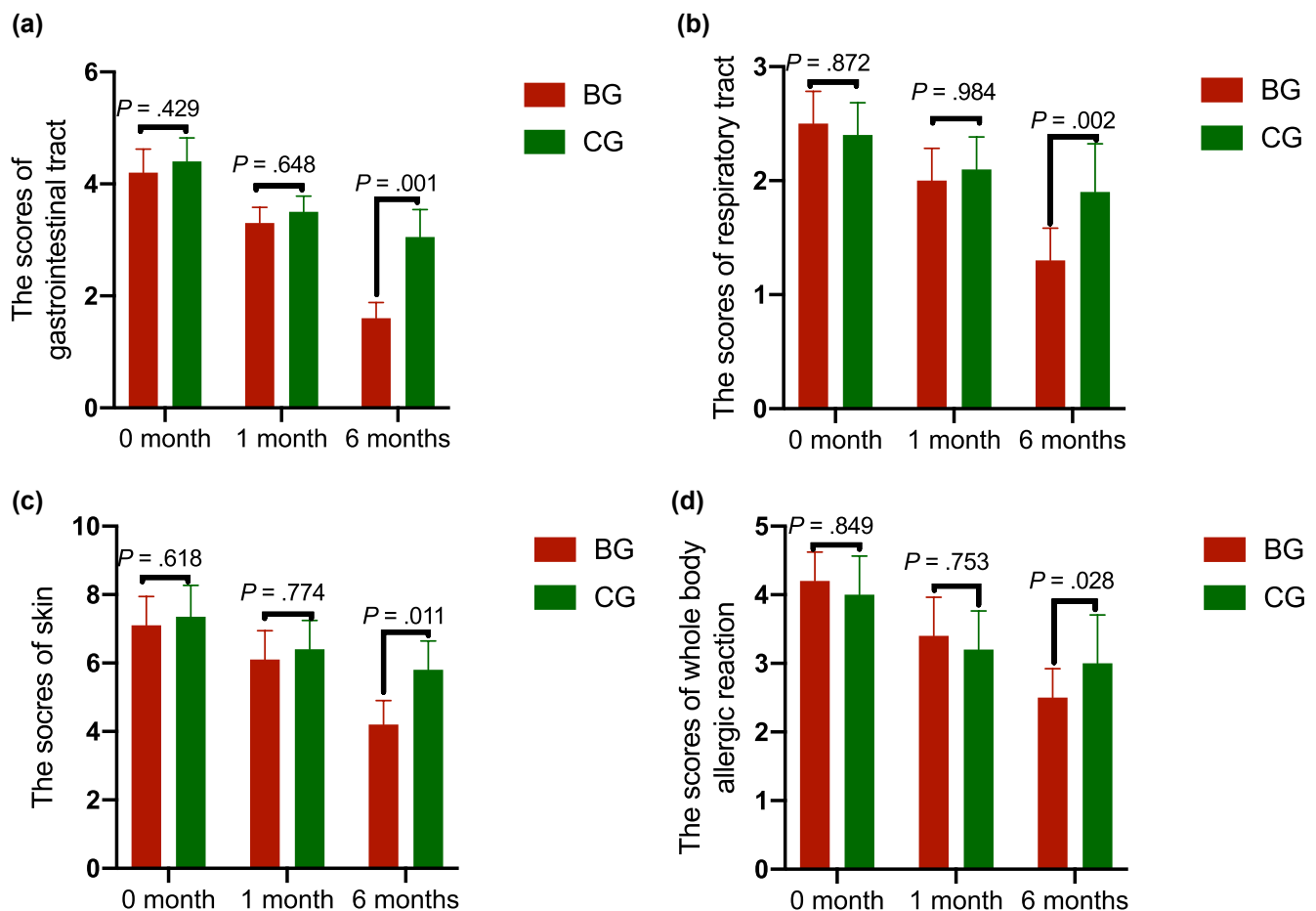


FIGURE 2 The comparison of allergic symptom scores between two groups. (a) The scores of gastrointestinal tract. (b) The scores of respiratory tract. (c) The scores of skin. (d) The scores of whole-body allergic reaction. $n = 123$ in the BG group and 121 in the CG group. The statistical difference was significant if $p < .05$

pets at home, mother's education, and family history of allergy were insignificant between the two groups (Table 1). These results indicate all these factors will not affect the CMPA risk in the follow-up. After 1-month follow-up, no patient was lost or uncontinuous for the present study. After 6-month follow-up, three cases could not be contacted and two cases lost their data, and thus, 123 subjects finished the present study in the intervention group. After 6-month follow-up, four cases could not be contacted and three cases lost their data, and thus, 121 subjects finished the present study in the control group. According to the above sample size calculation, the final sample size would not affect the present results. Among CMPA infants, the ratio of the male: female was 1.4:1. Premature, full-term, and overdue infants had 31 cases (12.11%), 209 cases (81.64%), and 14 cases (5.47%), respectively. Low-birth-weight, normal-birth-weight, and giant infants had 18 cases (7.03%), 193 cases (75.39%), and 45 cases (17.58%), respectively. Milk allergy was more likely to occur in the 2–4 months of age group, followed by the 4–6 months of age group. Among all the parameters, there was no statistical difference between the two groups (Table 1).

3.2 | Primary outcomes

The allergic symptom scores were similar between two groups at the primary timepoint for gastrointestinal tract (Figure 2a, $p = .648$), respiratory tract (Figure 2b, $p = .984$), skin (Figure 2c, $p = .774$), and whole-body allergic reaction (Figure 2d, $p = .753$). The statistical difference for the serum level of inflammatory cytokines TNF α

(Figure 3a, $p = .093$), IL-1 (Figure 3b, $p = .264$), and IL-6 (Figure 3c, $p = .532$), and anti-inflammatory cytokine IL-10 (Figure 3d, $p = .589$) was insignificant between two groups.

3.3 | Secondary outcomes

3.3.1 | *B. bifidum* TMC3115 reduced allergic scores

After 6-month follow-up, the digestive tract, skin, whole-body allergic reaction, and respiratory symptoms were relieved in the intervention group when compared with the control group after 6-month follow-up (Figure 2, $p < .05$). The allergic symptom scores were significantly different between the two groups at the primary timepoint for gastrointestinal tract (Figure 2a, $p = .001$), respiratory tract (Figure 2b, $p = .002$), skin (Figure 2c, $p = .011$), and whole-body allergic reaction (Figure 2d, $p = .028$). Three cases were still with skin rash and eczema in the control groups. There were still two cases of growth retardation, and weight and height were less than other infants. The secondary outcomes were improved in the intervention group when compared with the control group after 6-month follow-up (Figure 2, $p < .05$).

3.3.2 | *B. bifidum* TMC3115 increased anti-inflammatory properties in CMPA infants

After 6-month intervention of *B. bifidum* TMC3115, the serum levels of TNF α (Figure 3a), IL-1 (Figure 3b), and IL-6 (Figure 3c) were

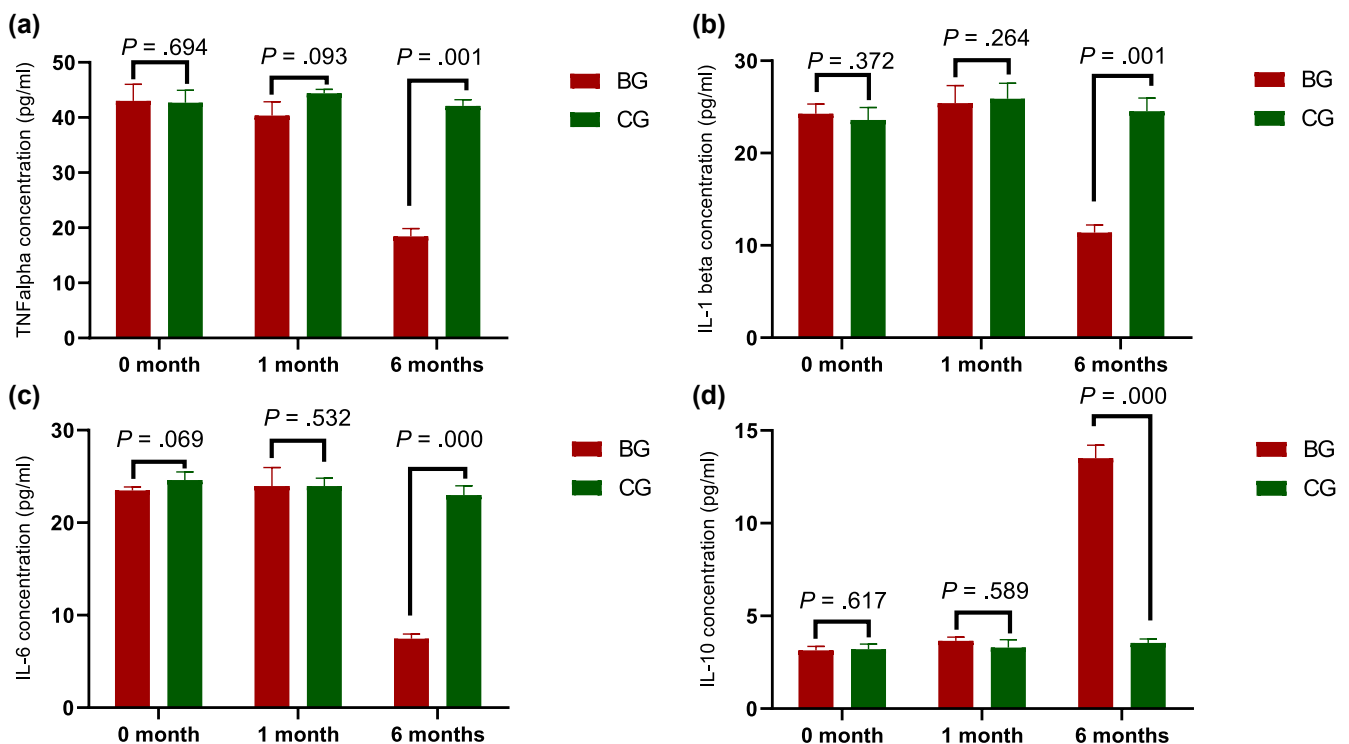


FIGURE 3 The effects of *Bifidobacterium bifidum* TMC3115 on the serum levels of inflammatory cytokines. (a) TNF α . (b) IL-1 β . (c) IL-6. (d) IL-10. * $p < .05$ versus the CG group. $n = 123$ in the BG group and 121 in the CG group

reduced and the level of IL-10 (Figure 3d) was increased when compared with those in the CG group ($p < .05$). The results suggested that *B. bifidum* TMC3115 increased anti-inflammatory activities in CMPA infants.

3.3.3 | *B. bifidum* TMC3115 reduced serum level of IgE and increased IgG2 in CMPA patients

Before probiotic intervention, the statistical difference for serum level of IgE was insignificant between the two groups (Figure 4a, $p = .258$). After *B. bifidum* TMC3115 consumption, the serum level of IgE in the *B. bifidum* TMC3115 group was lower than that in the control group (Figure 4b, $p = .001$). The results suggested that *B. bifidum* TMC3115 intake reduced serum level of IgE. In contrast, before the probiotic intervention, the statistical difference for serum level of IgG2 was insignificant between two groups (Figure 4c, $p = .654$).

After *B. bifidum* TMC3115 consumption, the serum level of IgG2 in the *B. bifidum* TMC3115 group was higher than in the control group (Figure 4d, $p = .017$). The results suggested that *B. bifidum* TMC3115 intake reduced serum level of IgE.

3.3.4 | *B. bifidum* TMC3115 ameliorated the allergy-associated side effects of CMPA infants

Before treatment, all the patients had skin, respiratory, crying, gastrointestinal symptoms, and growth retardation. No significant difference was observed for these symptoms between the two groups ($p > .05$). The onset time of these symptoms was from 2 to 4 weeks after birth and the duration was 2 weeks. Most of the infants in the intervention group showed improvement or disappearance of clinical symptoms within 8 weeks. Vomiting, diarrhea, constipation, and other gastrointestinal symptoms reduced from 1 to 2 weeks. Most

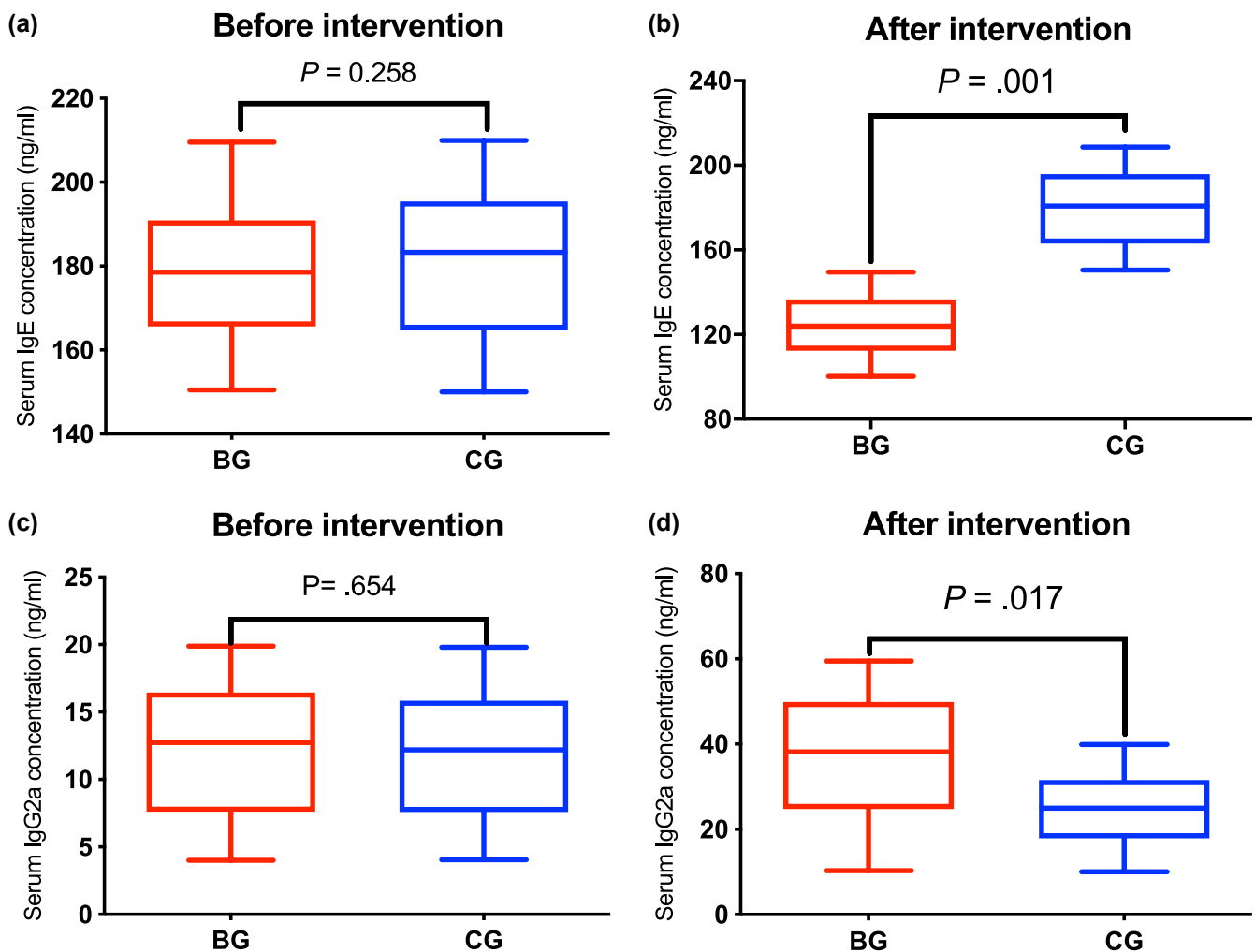


FIGURE 4 The effects of *Bifidobacterium bifidum* TMC3115 on serum levels of IgE and IgG2 in CMPA infants. (a) Serum levels of IgE before therapy. (b) Serum levels of IgE after therapy (secondary outcome). (c) Serum levels of IgG2 before therapy. (d) Serum levels of IgG2 after therapy (secondary outcome). BG, *Bifidobacterium bifidum* TMC3115 group and CG, placebo group. The statistical difference was significant if $p < .05$

of the respiratory symptoms, such as cough and asthma, disappeared from 2 to 4 weeks. Eczema reduced or disappeared within 2 to 8 weeks. Growth retardation was improved from 4 to 8 weeks. However, the clinical symptoms of the control group reoccurred, and they were difficult to be treated.

After 1 month of *B. bifidum* intervention, the total effective rate was insignificant between the two groups ($p > .05$) (Figure 5a). After 6 months of *B. bifidum* intervention, the total effective rate significantly increased in the intervention group when compared with the CG group ($p < .05$) (Figure 5b). Long-term *B. bifidum* intervention improved the therapeutic results.

3.3.5 | *B. bifidum* TMC3115 intervention improved gut microbiota

Chao1 indices showed that the mean microbial abundance in the BG group was higher than that in the CG group ($p < .05$, Wilcoxon rank-sum test). The majority of microbial diversity was found via Chao 1 (Figure 6a), observed species (Figure 6b), community diversity index (PD_whole_tree, Figure 6c), and Shannon indices (Figure 6d). Gut species abundance and uniformity in the CG group were lower than those in the BG group ($p < .05$, Figure 6d).

The profile of genus proportion of gut microbiota was similar between the two groups before and after 1-month intervention (Figure 7a). After 6 months of intervention, *B. bifidum* TMC3115 intervention increased the genus proportion of *Lactobacillus*, *Alistipes*, and *Barnesiella*, and reduced the proportion of *Anaerovibrio*, *Christensenellaceae*, *Oscillibacter*, *Bilophila*, *Dorea*, and *Roseburia* when compared with the CG group (Figure 7a). The genus proportion of *Bifidobacterium* significantly increased in the BG group after 6-month intervention (Figure 7a). The variety of genus proportion may be caused by the change of genus proportion since the change of *Bifidobacterium* species was not so significant after 1 month of *B. bifidum* TMC3115 intervention. Heatmap also showed the similar results (Figure 7b). The results suggest that *B. bifidum* TMC3115 treatment improved gut microbiota in the CMPA infants.

The statistical differences in microbial communities were compared between the two groups by using OTUs with the LEfSe analysis according to the previous report (Segata et al., 2011). The histogram features were presented as the LDA scores at the OTU level (Figure 8a). Taxa cladograms with LDA values > 2.0 were generated from the LEfSe analysis (Figure 8b). A total of 55 species of bacteria were abundant in the BG group and 45 species were abundant in the CG group. The diameter of each circle is proportional to its abundance. *Bifidobacterium*, *Lactobacillus*, *Turicibacter*, *Sutterella*, and *Parabacteroides* were the most predominant phylum in the BG group (Figure 8a,b), whereas the Firmicutes (*Anaerovibrio*, *Christensenellaceae*, *Oscillibacter*, *Bilophila*, *Dorea*, and *Roseburia*) were most dominant phyla in the CG group (Figure 8a,b).

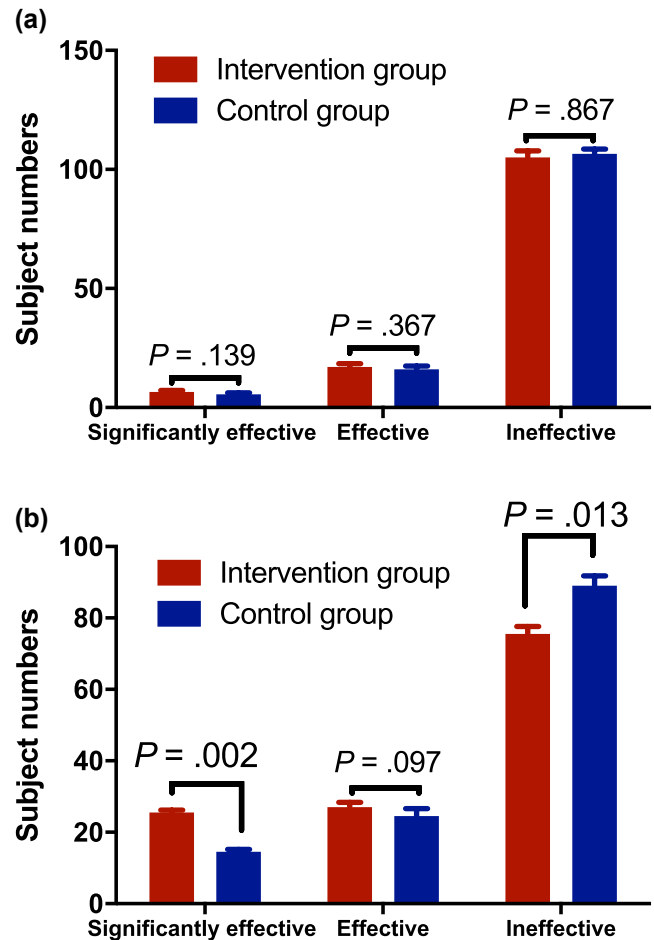


FIGURE 5 The therapeutic efficacy of *Bifidobacterium bifidum* TMC3115 for the infants with cow milk protein allergy (CMPA) between intervention group and control group. The therapy for CMPA was regarded as significantly effective if skin eczema was reduced by more than 80%. The therapeutic results were regarded as effective if skin eczema was reduced by 30%–80%, the therapy was regarded as invalid if the skin eczema was reduced by less than 30%. (a) The therapeutic results of *Bifidobacterium bifidum* TMC3115 for the infants with CMPA between intervention group and control group after 1 month. (b) The therapeutic results of *Bifidobacterium bifidum* TMC3115 for the infants with CMPA between intervention group and control group after 6 months. The statistical difference was significant if $p < .05$

3.4 | The levels of milk-specific IgE or IgG2 had a strong relationship with α -diversity scores of gut microbiota

Pearson's correlation coefficient test showed that the levels of milk-specific IgE had a strong positive relationship with α -diversity scores of gut microbiota (Figure 9a, Chao1; Figure 9b, observed species; Figure 9c, PD_whole_tree and Figure 9d, Shannon diversity index, $p < .001$). In contrast, the levels of milk-specific IgG2 had a strong negative relationship with α -diversity scores of gut microbiota (Figure 9e, Chao1; Figure 9f, observed species; Figure 9g, PD_whole_tree and Figure 9h, Shannon diversity index, $p < .001$).

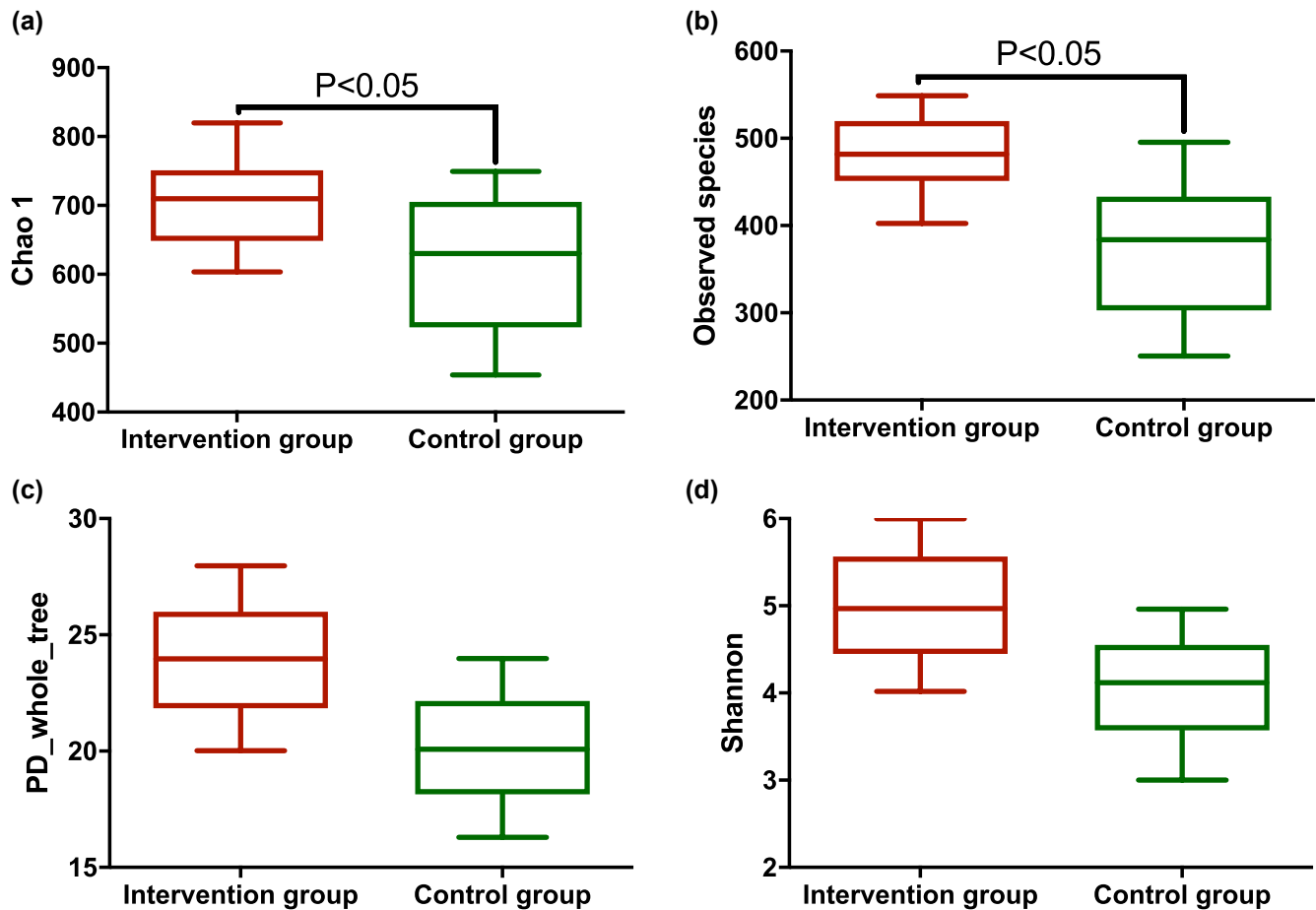


FIGURE 6 The α -diversity analysis of gut microbiota. (a) Chao1. (b) Observed species. (c) PD_whole tree and (d) Shannon diversity index. $^*p < .05$ versus the CG group. $n = 123$ in the BG group and 121 in the CG group

4 | DISCUSSION

Long-term *B. bifidum* TMC3115 consumption (6 months) reduced serum level of IgE, increased serum level of IgG2, improved the clinical symptoms of CMPA, anti-inflammatory responses, and secondary outcomes. Meanwhile, the genus proportion of *Lactobacillus*, *Alistipes*, and *Barnesiella* was increased while the proportion of *Anaerovibrio*, *Christensenellaceae*, *Oscillibacter*, *Bilophila*, *Dorea*, and *Roseburia* was reduced. All these results suggest that *B. bifidum* TMC3115 attenuates the symptoms of CMPA in the infants by possibly reducing IgE and increasing IgG2, and improving gut microbiota. Comparing with the present work, the effects of *B. bifidum* TMC3115 on allergy were mostly limited to animal study. Oral administration of *B. bifidum* TMC3115 in neonatal mice was found to attenuate IgE-mediated allergic risk in adulthood (RY Cheng et al., 2018). *B. bifidum* TMC3115 consumption in neonatal mice affected gut microbiota and immunity in adulthood (Cheng et al., 2019). However, the related clinical trials or the effects of *B. bifidum* TMC3115 on food allergy were seldom reported.

IgE is an important biomarker in the development of allergy (Gould & Sutton, 2008) and *B. bifidum* intervention reduced the level of IgE in the CMPA infants (Figure 3a). The results were consistent

with the previous report *Bifidobacterium* species inhibits IgE production in the immune modulation (Takahashi et al., 2006). Recent work also demonstrated that oral administration of *B. bifidum* TMC3115 may alleviate IgE-mediated allergic risk in an animal model (Cheng et al., 2018). On the contrary, IgG2 as IgG subclass may monitor allergen immunological activity (Movérare et al., 2017). *B. bifidum* TMC3115 intervention increased the serum level of IgG2, whereas the effects of *B. bifidum* TMC3115 intervention on IgG2 production was seldom reported. Much work is needed to confirm the present conclusion.

B. bifidum treatment especially increased in level of *Lactobacillus* species and lactic acid is the main metabolites of the probiotics (Tachedjian et al., 2017). Oral administration of nonpathogenic lactic acid bacteria to infants allows them to re-establish oral tolerance and plays an important role in the growth and maturation of the host's immune system and in immunomodulatory activities (Baglatzi et al., 2016; He et al., 2016). These differences reminded us that the changes in intestinal microbial flora were important approach in the prevention of food allergies (Rachid & Chatila, 2016). *Alistipes* and *Barnesiella* may be important probiotics in the maintenance the normal immunome responses (Kang & Cai, 2019). *Anaerovibrio*, *Christensenellaceae*, *Oscillibacter*,

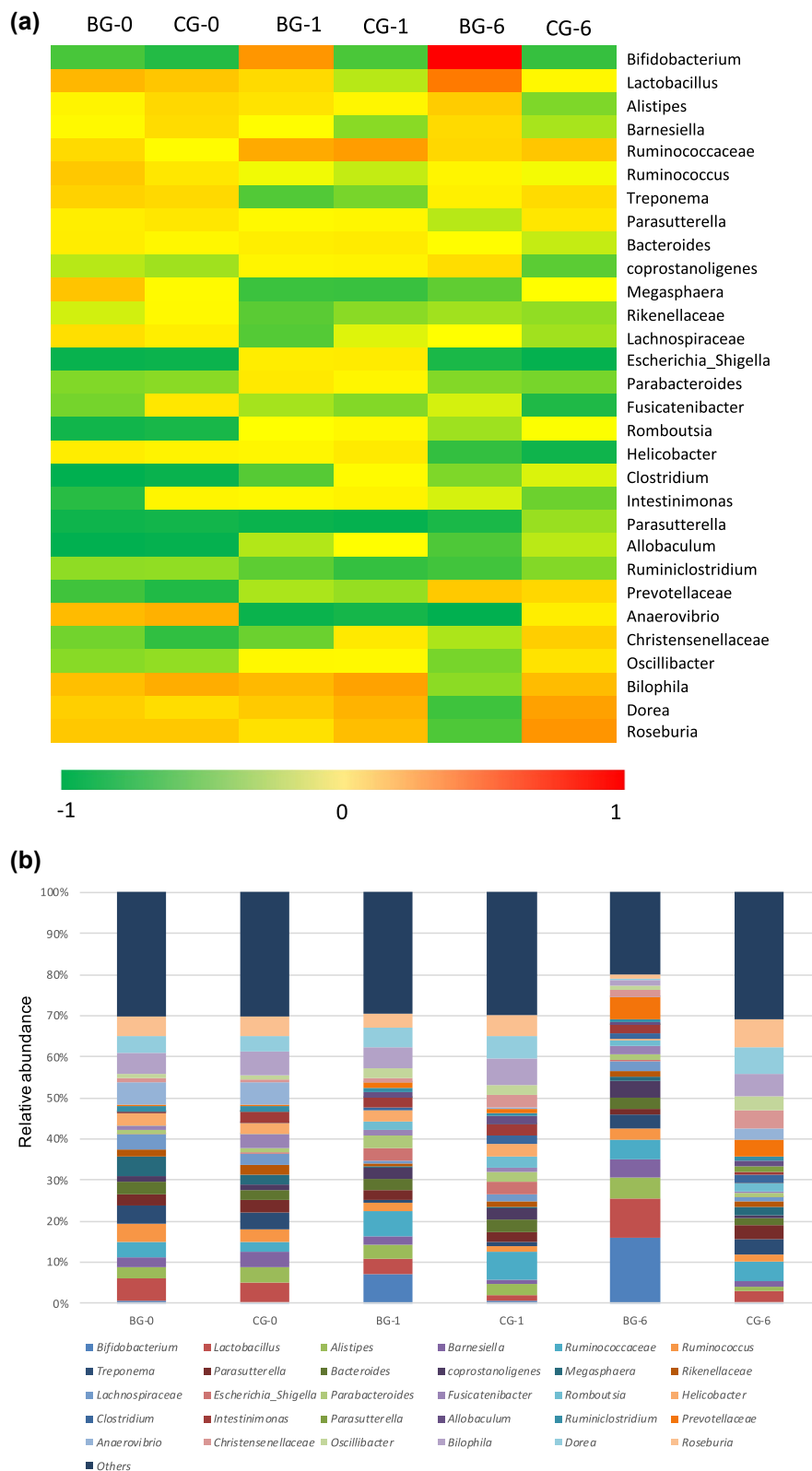


FIGURE 7 The genus proportion of gut microbiota between the two groups. (a) Bar column graph showed the changes of genus proportion of gut microbiota. (b) Heatmap showed relative abundance of the genus of gut microbiota between the two groups. 0, 1, and 6 stands for before, and after 1- and 6-month intervention

Bilophila, *Dorea*, and *Roseburia* may company with allergy diseases, which can be controlled by long-term *B. bifidum* consumption. The anti-allergic properties of *B. bifidum* TMC3115 may be involved

with many aspects. Food allergy may be caused by gut microbiota composition and function, and gut microbiota dysbiosis. *B. bifidum* TMC3115 was found to control gut dysbiosis and constipation by

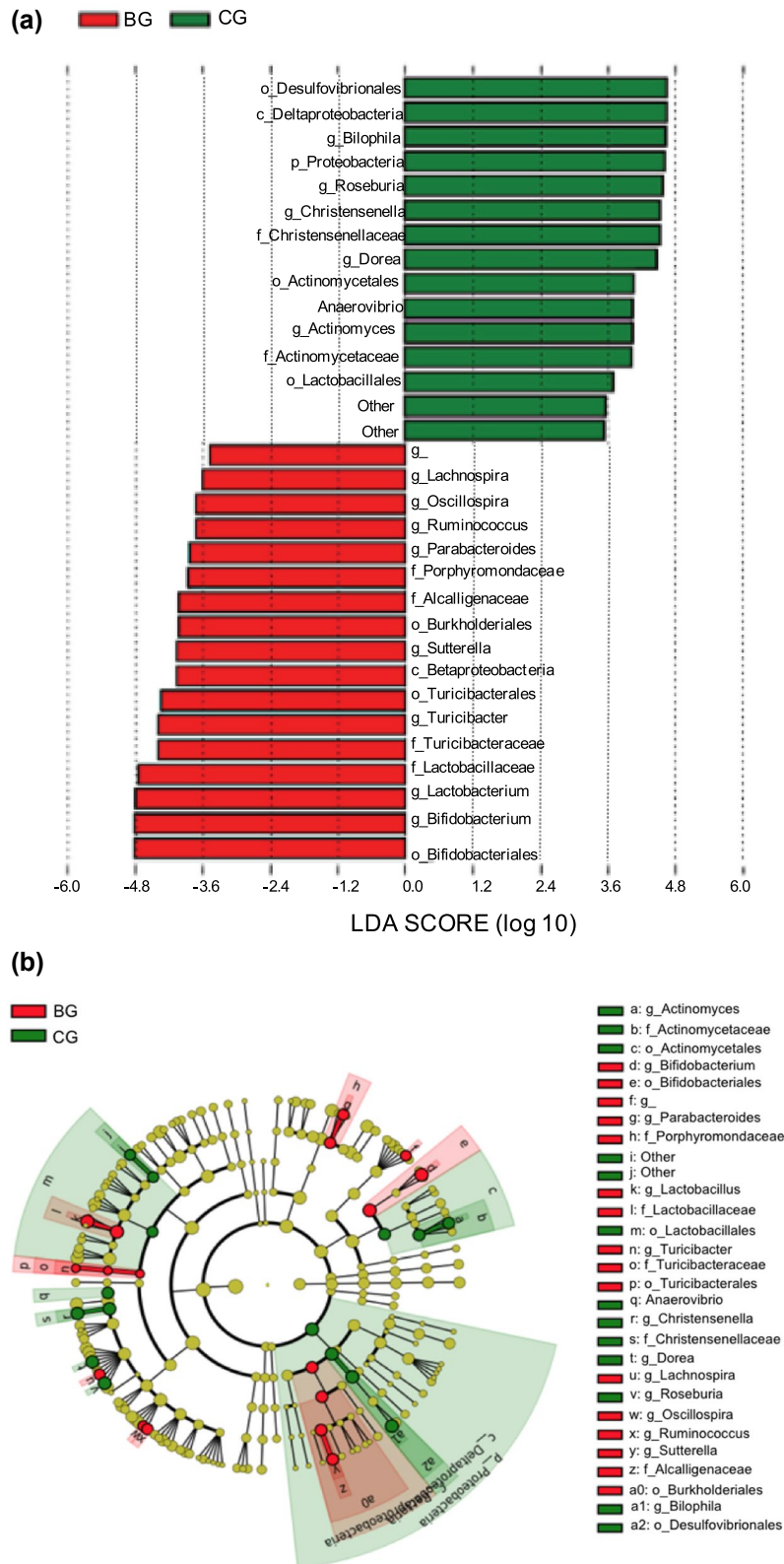


FIGURE 8 Different structures of gut microbiota between the BG and CG groups. (a) The taxa discriminated among the two groups based on linear discriminant analysis (LDA) effect size method. (b) Cladogram depicting the phylogenetic distribution of gut microbiota between the two groups. The diameter of the circle was proportional to the relative abundance (the levels were arranged from the inner to outer rings as genus, family, order, class, and phylum)

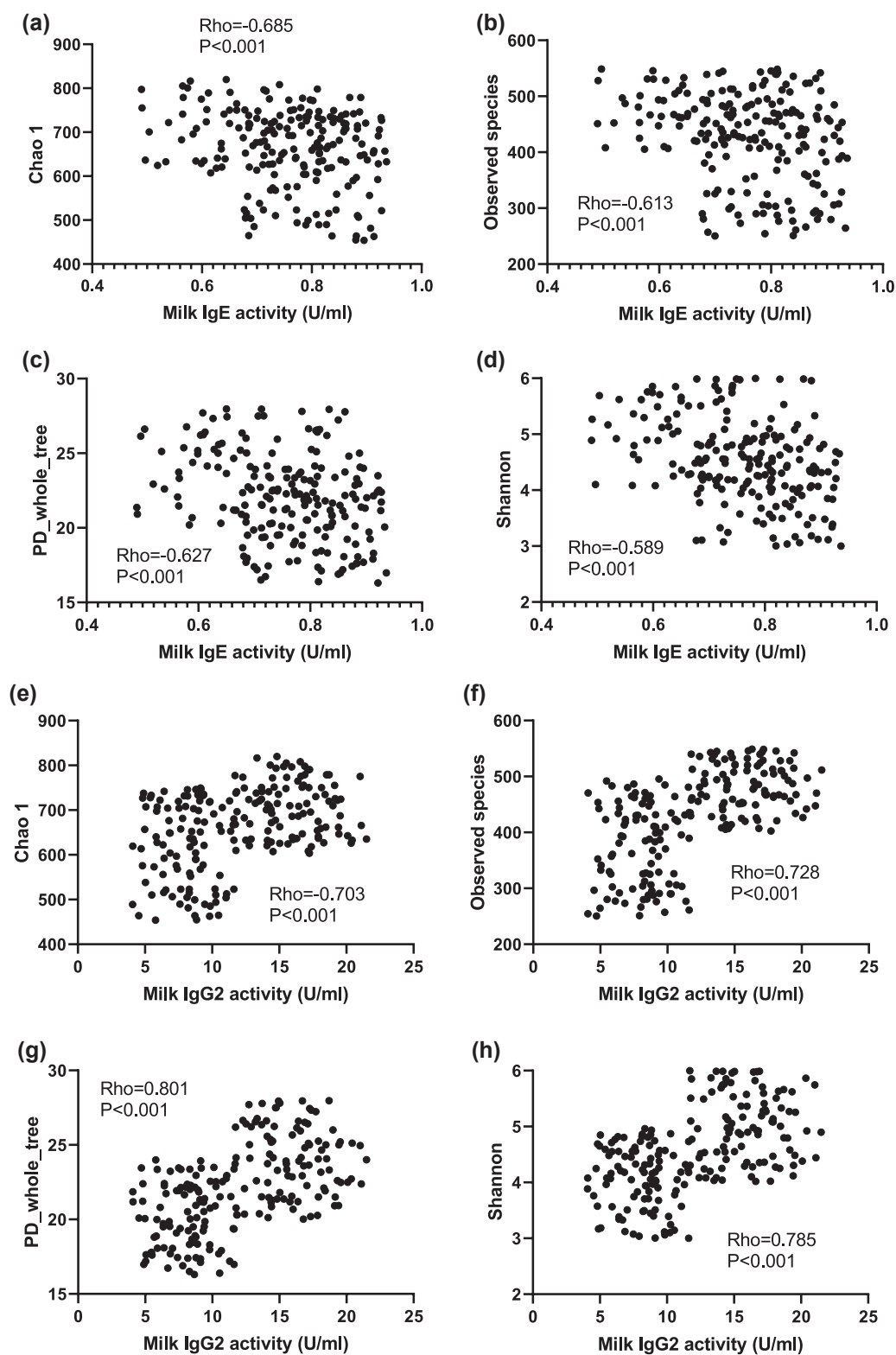


FIGURE 9 Pearson's correlation coefficient test between the levels of milk-specific IgE or IgG, and α -diversity scores of gut microbiota. (a) IgE and Chao1. (b) IgE and observed species. (c) IgE and PD_whole_tree. (d) IgE and Shannon diversity index. (e) IgG and Chao 1. (f) IgG and observed species. (g) IgG and PD_whole_tree. (h) IgG and Shannon diversity index

improving the intestinal environment, supporting its potential application in the treatment of food allergy.

Allergen has been well-defined by the International Union of Immunological Societies (IUIS) as being molecules that essentially induce IgE-mediated atopic allergy in humans with a prevalence of IgE activity above 5% (Troiano, 2016). Gut microbial dysbiosis has been found to be closely accompanying with allergen-specific IgE responses in kids with airway allergy (Chiu et al., 2019). The results suggest gut microbial composition should be related to atopic allergy risk. An increase in Enterobacteriaceae species numbers has been informed in several gut microbiota-associated inflammatory diseases, including atopic allergy, indicating the critical role of gut microbiota in atopic allergy. A clinical trial also demonstrates that the probiotics (*Lactobacillus salivarius*, *Lactobacillus paracasei*, *Bifidobacterium animalis subspecies lactis*, and *Bifidobacterium bifidum*) also reduce the occurrence of atopic eczema in premature children (Allen et al., 2014).

There were some limitations to the present experiment: (a) all the patients were from the same city and would cause a potential bias for the present study; (b) the sample size was still small, and the experimental subjects were from a larger population would be needed to confirm the present results; (c) comparing with the previous report (Liu et al., 2018), only milk allergy was explored in the present study. *B. bifidum* TMC3115 has gotten much attention for possible health benefits. *B. bifidum* TMC3115 exerted greater antimicrobial activity than *B. lactis* (Tahamtan & Sarvestani, 2016); (d) *B. bifidum* TMC3115 was more effective than *B. lactis* at colonizing the fecal *Bifidobacterium* in milk-fed infants (data were not shown). However, the exact comparison could not be performed because of the difference of baseline characteristics including different inclusion and exclusion between previous and the present studies; (e) gut microbial dysbiosis has been found to be closely accompanying with atopic allergy. Unfortunately, the relationship between the gut microbiota composition and CMPA was not explored by using an animal model in the present work and (6) due to the short period of the study, the related risks of milk protein allergy in infant with allergic rhinitis, asthma, and other allergic diseases, could not be fully assessed, and nutritional intervention remained to be further explored.

5 | CONCLUSION

For the infants with CMPA, *B. bifidum* TMC3115 is potential probiotics to eliminate allergic symptoms. *B. bifidum* TMC3115 treatment is very safe and effective for the therapy of food allergy. *B. bifidum* TMC3115 ameliorated milk protein allergy in the infants by regulating gut microbiota, and should be developed a potential way in the prevention of infant milk allergy.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTION

Wei Jing: Conceptualization; Data curation; Writing-review & editing. **Qingbin Liu:** Conceptualization; Data curation; Formal analysis; Methodology; Resources; Software; Visualization; Writing-original draft. **Wei Wang:** Conceptualization; Formal analysis; Funding acquisition; Methodology; Supervision; Visualization; Writing-original draft; Writing-review & editing.

ETHICAL STATEMENT

The trial register No. is ChiCTR1900026918 and registered on October 26, 2019. Before the present experiment, all procedures were approved by the human research committee of Affiliated Hospital of Changchun University of Traditional Chinese Medicine (Approval No. CCZYFYLL2018) and the research has been carried out in accordance with the World Medical Association Declaration of Helsinki. Informed written consent was obtained from each infant's guardian.

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