Clinical trial protocol

Effect of adding breast milk oligosaccharides and multinutrient optimized infant formula on the growth and development of infants aged 0 to 1 years: a multicenter, double-blind, randomized, controlled trial

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catalogue

1 Summary of the clinical trial protocol

Research name	Effect of adding breast milk oligosaccharides and multinutrient optimized infant formula on the growth and development of infants aged 0 to 1 years: a multicenter, double-blind, randomized, controlled trial
research design	Multicenter, randomized, controlled, parallel, double-blind trial
sample capacity	240
Intervention cycle	360 Days
purpose of research	Compare add milk oligosaccharides and a variety of nutrients optimization of infant formula and conventional infant formula on the immune function of infants under 1 year of age, and compared with breastfeeding infants, explore different infant formula on infant intestinal health, immune function, growth and development, cognitive development and tolerance.
Main end point	Intestinal indicators (16s r RNA, SCFAs)
Secondary end point	· Inflammation indicators: serum hsCRP; • Nutritional index: serum albumin; • Immune indicators: fecal sIgA and blood lymphocyte subsets; · Growth and development indicators: height, weight, head circumference and chest circumference; · Bone development indicators: osteocalcin, alkaline phosphatase; • Tolerance indicators: survey of infant gastrointestinal comfort, infant sleep crying, infant diarrhea and upper respiratory tract infection, infant stool Bristol stool classification; · Cognitive development: Bailey Infant Development Scale (version

	IV), serum amino acids.
Safety indicators	• Adverse events and serious adverse events.
Inclusion criteria	Inclusion criteria for the breast-feeding group: · The 0 ~ 28 days after birth; · Single-child birth; · The gestational age was from 37 to 42 weeks; · Birth weight is 2500 ~ 4000 g; · Parents or guardians agree to exclude the infant from participating in other interventional clinical studies during this study; · Parents or guardians agreed to feed the baby according to the trial protocol; Inclusion criteria for the artificial feeding group: · The 0 ~ 28 days after birth; · Single-child birth; · The gestational age was from 37 to 42 weeks; · Birth weight is 2500 ~ 4000 g; · Parents or guardians agree to exclude the infant from participating in other interventional clinical studies during this study; · Parents or guardians agreed to feed the baby according to the trial protocol; · The mothers cannot be exclusively breastfeeding due to subjective and objective reasons.
Exclusion criteria	· Artificial assisted reproduction; · The mother suffers from diseases that may endanger the intrauterine growth; · The mother had gestational diabetes mellitus or severe metabolic disease or chronic disease at the time of pregnancy; · Having congenital malformations and genetic, chronic, and

	congenital diseases that may interfere with the investigation; · IgE mediated milk protein allergy or factors that increase the risk of milk protein allergy; · Having an acute infection or gastroenteritis; · Functional gastrointestinal diseases, such as gastroparesis; · Is participating in other clinical trials; · The investigator was unable to determine the parents' willingness or ability to comply with the protocol requirements.
Exit standard	• Ineligible (ignored during screening, occurred during the study or found by review); • Serious protocol deviation occurs; • Serious non to protocol or test requirements; • Need to terminate the designated formula due to adverse events or cause failure to continue following the trial procedures; • Need to discontinue the designated formula or cause the inability to follow the trial procedures due to disease progression; • Withdraw informed consent or remove informed consent; • Misvisit.
Intervention method	Test group: Artificial feeding of the experimental formula 0-4 months of age: artificial feeding is not less than 2 times a day; 4~12 months of age: artificial feeding is not less than 5 times a day; Control group: artificial feeding of the control formula 0-4 months of age: artificial feeding is not less than 2 times a day; 4~12 months of age: artificial feeding is not less than 5 times a day; Breastfeeding group: breastfeeding 0-4 months of age: exclusive breastfeeding (must be fully

<table>
<tr><td></td><td>breastfeeding 1 day before the visit)
4 to 12 months old: breastfeeding at least once a day</td></tr>
<tr><td>Research implementation</td><td>In the artificial feeding group, 160 infants from 0 to 28 days were randomly divided into trial group and control group, 80 infants in each group; 80 breastfed infants were included in the same period. The test group, control group and breastfeeding group were fed the infants. Based on the time of the last visit, the visit was conducted at 28,90 ± 3 days, 180 ± 5 days and 360 ± 7 days after birth.
<table>
<tr><th>metric</th><th>dressing by screening</th><th>Visit 1 (28 Days)</th><th>Visit 2 (90 Days)</th><th>Visit 3 (180 Days)</th><th>Visit 4 (360 Days)</th></tr>
<tr><td>Demographic data</td><td>√</td><td>√</td><td></td><td></td><td></td></tr>
<tr><td>Bailey Infant Development Scale (Version IV)</td><td></td><td></td><td></td><td></td><td>√</td></tr>
<tr><td>Growth and development indicators</td><td></td><td>√</td><td>√</td><td>√</td><td>√</td></tr>
<tr><td>excrement and urine
• 16S rRNA
· SCFAs
· sIgA</td><td></td><td>√</td><td></td><td>√</td><td>√</td></tr>
<tr><td>blood
· hsCRP
• Lymphocyte subsets
• amino acid
· bone gla protein
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	· albumin					
	Feeding status, Intake questionnaire (breastfeeding and formula feeding)		√	√	√	√
	Gastrointestinal comfort questionnaire		√	√	√	√
	Sleep crying questionnaire		√	√	√	√
	Questionnaire for diarrhea and upper respiratory tract infections		√	√	√	√
	Bristol Stool Classification		√	√	√	√

2 research background

Breast milk is widely recognized as the most perfect food for the newborn period. Breast milk naturally contains the best proportion of various nutrients suitable for the baby's digestion and absorption[,]12, Such as protein, fats, carbohydrates, vitamins, and minerals. In addition, breast milk also contains a variety of bioactive ingredients suitable for infants, such as lactoferrin, immunoglobulin, hormones, oligosaccharides, probiotics and so on[,]34, These bioactive ingredients play an important role in the health of infants and young children.

2.1 Breast milk oligosaccharides

Breast milk oligosaccharides (Human Milk Oligosaccharides, HMOs) are a natural bioactive substance in breast milk, second only to lactose and fat, is the third largest solid component of breast milk, and have important physiological functions. Studies showed that the content of HMOs in colostrum was 20 to 23 g / L and decreased to 12 – 14 g / L in mature milk[]5, Higher levels of HMOs in breast milk than in term mothers[]6. Traditional infant formula does not add HMOs, but dietary fiber such as galactose (GOS) and fructose oligosaccharides (FOS) are used as alternatives[,]78. The HMOs are produced by D-glucose (D-glucose, Glc), D-galactose (D-galactose, Gal), N-acetylglucosamine (N-acetylglucosamine, GlcNAc), L-fucinose (L-fucose, Fuc) and sialic acid (sialic acid, Sia) and other five monosaccharides constitute complex glycans, Each HMOs contains a single lactose (Gal- β -1, 4-Glc) at the reducing terminus, And based on this, with β -1, 3 or β -1, 6-bond linkage to galactose β -1, 3-N-acetylglucosamine (Gal- β -1, 3-GlcNAc, This disaccharide structure is also known as the lacto-N-biose, Or type 1 chain structure) or N-acetylylactose (N-acetyllactosamine, Gal-β-1, 4-GIcNAc, Type 2 chain structure) of the extended sugar chain, Formation of a lacto-N-tetrasaccharide (lacto-N-tetrose, LNT), lactol-N-new tetrasaccharide (lacto-N-neotetraose, LNnT), lactol-N-

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hexasaccharide (lacto-N-hexaose, LNH) and other core structures. The structure of FOS and GOS is simpler, and the addition of FOS and GOS in formula is considered a relatively reasonable and cheap scheme to simulate breast milk. The difference between these two and HMOs in breast milk still need more research to demonstrate. The 2 '-fucinose-based lactose (2' FL) and LNnT have been used in infant formula. Some studies showed that supplementing 2 ′ -FL (1 g / L) and LNnT (0.5 g / L) on the basis of formula, the intestinal flora of infants at 3 months contained more bifidobacterium and less Echia, digestive Streptococcus, closer to that of breast-fed infants; the content of important metabolites such as propionate, butyrate and lactate in feces of infants where HMOs were supplements[,]910。

HMOs are capable of blocking the adhesion of pathogens. Many viruses or bacteria must attach to the surface of epithelial cells to proliferate and cause disease, and HMOs act as soluble ligands like blocking adhesion to pathogens. Animal experiments showed that 2 ′ FL reduced the invasion of Chlamydia jejunae by 80% and inhibited the release of mucosal pro-inflammatory signals, which subsequently reduced the number of episodes of diarrhea. LNnT was shown to reduce the number of S. pneumoniae cells in the lungs of an animal model[]11. The anti-adhesive properties of HMOs also apply for dissolution. Entamoeba, and histolytica, capable of destroying epithelial cells of the large intestine, liver, lung or spleen, is regarded as the third leading cause of parasite death worldwide[]12. Entamoeba histolytica needs to be attached to the colon mucosa of the host, and endohistolytica will be excreted with the feces and will not cause disease.

HMOs are able to help establish and maintain a healthy intestinal flora in infants. From 0 to 3 years of age, the formation of intestinal flora is a developing process. The bacteria in the gut mainly include Escherichia coli, Klebsiella, Enterobacter, Bacteroides and Clostridium spp. Bifidobacterium dominated the gut of breastfed infants, with Clostridium and Enterococcus, and Klebsiella and Enterobacteria being the least. The gut flora of artificially fed infants is more similar to the adult digestive tract, and the composition is more complex than that in breast-fed infants. Bifidobacterium should dominate in the healthy intestinal flora of infants. In recent years, people have increased the content of bifidobacterium in the intestine by adding

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prebiotics to formula. HMOs are not destroyed by the gastric acid of the human body, nor decomposed by digestive enzymes, and can directly reach the large intestine, stimulate the growth of beneficial bacteria (bifidobacteria and Lactobacillus) in the intestinal tract, indirectly inhibit the growth of harmful bacteria, and maintain the balance of intestinal microecology. Therefore, HMOs are regarded as the primary prebiotics (prebiotics) of human beings[]13. Bifidobacterium in the large intestine can produce acetic acid, propionate and butyric acid through fermentation, acetic acid can reduce the pH value in the intestinal tract and inhibit the growth of pathogenic bacteria; propionate and butyrate have important physiological functions; butyrate is an important energy source for colon cells. HMOs are able to indirectly increase the production of short-chain fatty acids (SCFA s), and most SCFAs are rapidly absorbed by colonic cells or used as energy sources[]14. Research evidence suggests that SCFAs are able to participate in the regulation of gene expression[]15, Play an important role in the activation and differentiation of immune cells and is associated with inflammatory and allergic diseases[,]1617。

HMOs are able to provide protection against multiple viruses[„]181920. HMOs promote the maturation of the immune system, produce a more balanced Th 1 / Th 2 cytokine response; stimulate the immune response and maturation of epithelial cells to protect the host from viral infection; affect the diversity and concentration of the microbiome and stimulate the growth of commensal bacteria. Another antiviral activity of HMOs is based on structural similarity to the glycochains of glycoconjugates present on the epithelial surface, mimicking the surface glycans of epithelial cells, and in turn capturing viruses that do not adhere to the cells. Virus lectin receptors blocked by HMOs fail to participate in recognizing the glycosites

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present on the host cell surface, thereby preventing their adhesion and colonization. Against rotavirus and norovirus infections, HMOs have been found to be useful as an alternative to current therapeutic approaches. HA may be used in humans as a HMOs-based drug to treat influenza. Monosialylated HMOs (3 ′-SL and 6 ′-SL) have been shown to reduce influenza infection in cell culture assays.

HMOs are able to promote immune function development in infants. In the first 6 months of life, breastfeeding was shown to promote the maturation of the immune system, feeding enough to reduce the incidence of many diseases such as asthma, allergies, inflammatory bowel disease, type 1 diabetes, celiac disease, and leukemia. HMOs are very important components in breast milk and can affect the infant immune system through multiple mechanisms including blocking pathogen adhesion and promoting bifidobacterium colonization. The composition of the infant gut flora and epithelial cell responses mediated by HMOs may indirectly affect the infant's immune system. In vitro studies have shown that HMOs can also directly modulate the immune response. The HMOs may act locally on the lymphohistiocytes associated with the mucosa[,]2122. Many immune receptors recognize the oligosaccharide structures of their glycoprotein ligands. HMOs are structurally similar to ligand, thus it is speculated that they may bind directly to immune cells[,]2324, This binding can cause signaling, leading to changes in the nature and function of immune cells.

HMOs can reduce the risk and mortality of necrotizing enterocolitis (NEC). NEC causes severe and fatal destruction of the gut in infants, with more than 25% of NEC babies dying, and surviving infants often have long-term neurological complications. The risks of NEC were 6 to 10 times lower than formula-fed infants[,]2526. Animal experiments in rat models showed that HMOs prevent the release of NEC. If these results are translated into NEC in humans, lactose disialate-N-tetrasaccharide (DSLNT) can be used to prevent or treat NEC in formula-fed infants, and its concentration in breast milk can be used as a biomarker to identify the risk of breast-

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fed infants with the disease. It may also facilitate the development of innovative therapies for NEC[,]2728。

The HMOs are able to promote the early development of the infant brain. Parts of the HMOs in breast milk are terminal to adhere to high concentrations of sialic acids. Sialic acid is involved in the composition of gangliosides and glycoproteins in human brain tissue, and is closely related to nerve synapses and nerve conduction, so breastfeeding helps to enhance nerve synaptogenesis and promote the development of infant nervous system[]29。

2.2 lactoferrin

Lactoferrin is an iron-bound glycoprotein with a variety of physiological functions, with a molecular mass of about 70 – 80 kilodaltons[]30. Lactoferrin is widely distributed in the human body, in milk, saliva, tears, nasal secretions, gastrointestinal fluid, urine, semen and other external secretions, among which lactoferrin is the most abundant in breast milk[,,]313233. Lactoferrin content in the mature milk of term and preterm infants ranged from 1.25 to 4.59 mg/mL and 1.6 to 6.59 mg / mL, respectively[,,,]34353637，

Lactoferrin has many physiological and biological functions, especially in terms of immune function has been widely recognized. Lactoferrin for many bacteria have

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different degrees of antibacterial effect, with broad spectrum antibacterial, it may benefit from the following mechanism: (1) the antibacterial properties of lactoferrin from its high iron binding ability, and the growth process of bacteria also need a lot of iron, lactoferrin can compete and inhibit the use of iron source, achieve the purpose of antibacterial[]38. (2) for gram-negative bacteria, lactoferrin by positive amino acid group and bacterial outer membrane anionic lipopolysaccharide (LPS), can inhibit LPS and other cations (such as Ca 2 +, Mg2 +, etc.), and cause LPS from the cell wall, bacterial cell wall permeability increase, bacterial cell apoptosis, achieve the effect of antibacterial, research also proved that the interaction of lactoferrin and LPS can also enhance natural antibacterial agents (such as lysozyme) antibacterial[,,]394041. (3) For Gram-positive bacteria, the positive group of lactoferrin can react with the negative charge brought by the anionic material lipoteichoic acid on the surface of the bacteria, thus reducing the negative charge of the cell wall, leading to the reaction of lysozyme and the underlying peptidoglycan, and playing the inhibitory role of lysozyme[]42。

Recent studies have found that lactoferrin has antiviral ability, which may be achieved through the following ways: (1) lactoferrin interferes with the viral replication and syncytial process to inhibit the activity of the virus. In vitro results show that lactoferrin inhibits viral replication in host cells in human plasma and milk, thereby inhibiting AIDS (AIDS) viral activity[]43. (2) Lactoferrin can inhibit the entry of viral particles into the host cells by either directly attaching to the virus or by blocking its cellular receptors. The results show that lactoferrin can interfere with the transmission of human immunodeficiency virus (HIV-1), blocking its transmission from dendritic cells to TCD 4 cells by blocking the binding of the virus to epithelial cellshand over[]44。

Moreover, lactoferrin can play an immunomodulatory role by acting on immune

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cells and cytokines. The results showed that the treatment of recombinant human lactoferrin (rhLF) vin-120 in enteritis mice increased the number of regulatory T cells and activated regulatory T cells (Tregs) to secrete IL-17 to relieve inflammation, and tested the effect of rh LF on CD4 + cells in vitro, which also confirmed that rh LF can induce the differentiation of CD4 + T cells into Tregs, leading to an increase in the number of Tregs[]45. In addition, lactoferrin enhances the cytotoxic function of NK cells and lymphokine-activated killer (LAK) cells[]46. lactoferrinCan directly bind to the lipidA of LPS embedded in the bacterial surface, And to promote its release, Disrupt the stability and permeability of the bacterial outer membrane; Simultaneously in the cells, Free LPS can also bind to sCD 14 on the cell membrane, The activation of the Toll-like receptor (TLR) 4 signaling pathway to increase the generation of pro-inflammatory mediators; After the competitive binding of LPS and / or sCD 14 by lactoferrin, Thus reducing the binding of LPS-sCD 14, Blocking the activation of the TLR-4 signaling pathway and signaling, A decrease in the secretion of pro-inflammatory factors; The LF-LPS complex may activate TLR 4 signaling, Leading to the expression of the proinflammatory components[]47. Lactoferrin can promote IL-2 synthesis in macrophages, and IL-2 can guide macrophage migration to sites of inflammation and activate CD4 + T lymphocytes[]48。

2.3 Docosahexaenoic acid and eicosapentetraenoic acid

Docosahexaenoic acid and eicosapentetracenoic acid is two important long chain polyunsaturated fatty acid (LCPUFA), referred to as DHA and ARA, can be the precursor fatty acid α -linolenic acid (ALA) and linoleic acid (LA) in the body, the two is not only constitute two main fatty acids of brain phospholipids, or the central nervous system development essential nutrients, DHA accounted for 10% of the brain. It is known that the critical period of brain development is from the beginning of pregnancy to the second 2 years of birth. During this critical period of development, brain cells develop and differentiate rapidly, and are extremely sensitive to internal and external environment changes and stimulation. Dobbing Sands showed that the weight of the brain increased from about 20g in the second to third months of life to

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about 1200g at 2 years[]49. Martinez et al reported a nearly 30-fold increase in DHA and ARA content in infant brains from birth to 2 y[,]5051. This also reflects the importance of DHA and ARA in the growth and development of infants and young children.

Although fetuses and newborns can also synthesize DHA and ARA by themselves, these long-chain polyunsaturated fatty acids are not able to meet the needs of rapid growth[]52. Therefore, early in life growth and development, breast milk and or breast milk substitutes are the main food sources for DHA and ARA. Brenna The team analyzed 106 breast milk studies, which selected 65 studies with a total sample size of 2474 women and found that the mean content of DHA (% ± standard deviation) was 0.32 ± 0.22% total fatty acids (range: 0.06~1.4%) and ARA was 0.47 ± 0.13% total fatty acids (range: 0.24-1.0%). DHA is less abundant in breast milk than ARA and more different than ARA. In addition, the results found that the coastal population had the highest DHA content and was associated with more dietary intake of seafood, and the lowest DHA content occurred in inland populations and developed countries, which was associated with low fish intake[]53. Yuhas et al collected 440 breast milk samples from Australia, Canada, Chile, China, Japan, Mexico, Philippines, the United States and showed that DHA ranged from 0.17 wt% to 0.99 wt%, the highest value was Japan, the lowest value was samples from Canada and the United States, and the average DHA content of samples from China was 0.83%. The content of ARA was stable in several studied countries, from 0.36wt%~0.49wt%, the mean was 0.41%. The result in China was 0.49% total fatty acids. The mean ± standard deviation of the absolute content of Chinese DHA is 149.8±10.5mg/L, and the mean ± standard deviation of the absolute content of ARA was 207.8 ± 12.05 mg / L[]54. Koletzko B Led his team to review LCPUFA during pregnancy, lactation and infancy, and showed that the overall average of ARA in breast milk ranged from 0.35% to 0.70% total fatty acids, and the overall mean of DHA ranged from 0.17% to 1.0% total fatty acids. Based on the results of the literature study, it is recommended that the amount of DHA is 0.2 to 0.5%, and the minimum amount of ARA should not be lower

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[49] Dobbing J, Sands J, Quantitative growth and development of human brain[J].Arch.Dis.Child, 1973, 48:757-767.

[50] Martinez M, Tissue levels of polyunsaturated fatty acids during early human development [J].J Pediatr.1992, 120(4):S129-38.

[51] Martinez M, Mougan I, Fatty acid composition of human brain phospholipids during normal development [J].J.Neurochem.1998, 71:2528-2533.

[52] Lauritzen L, Hansen HS, et al.The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina.Progress in Lipid Research, 2001, 40:1-94.

[53] Brenna J T, Varamini B, Jensen RG, et al.Docosahexaenoic and arachidonic acid concentrations in humanbreast milk worldwide [J].Am.J.Clin.Nutr.2007, 85(6): 1457-1464.

[54] Yuhas R, Pramuk K, Lien EL, Human Milk Fatty Acid Composition from Nine Countries Varies Most in DHA.Lipids, 2006, 41(9):851-858.

than the DHA content[]55. A study of immune factors and fatty acids between the University of Reading and the Chinese Center for Disease Control and Prevention in three different geographical areas in China (Urwin HJ, 2013), Healthy singleton women from 18 to 22 weeks of pregnancy in Jurong, Jiangsu (river and lake region), Xushui (inland area) and Rizhao (coastal area), A cross-sectional study collected colostrum (3 to 5 days), transitional milk (14 days) and mature milk (28 days) for compositional analysis, See the table below, Higher levels of DHA and ARA in breast milk are associated with higher levels of immune factors in breast milk, It suggests that these fatty acids can promote the maturation of the infant intestine and the immune system[]56. The expert group led by the Chinese Nutrition Society combined 14 domestic reports on DHA and ARA content of breast milk from 1995 to 2013, and concluded that the median content of DHA of Chinese mothers was 0.42% total fatty acids, and the median content of ARA was 0.61% total fatty acids.

delspray	fatalism	River and lake area	foreland	boo-ay
ARA	3～5	0.94%	0.85%	0.88%
	14	0.85%	0.80%	0.82%
	28	0.76%	0.71%	0.70%
DHA	3～5	0.66%	0.61%	0.41%
	14	0.53%	0.53%	0.35%
	28	0.50%	0.55%	0.27%

Based on the data of numerous breast milk studies at home and abroad and the daily fatty acid intake of infants, the recommended intake of DHA and ARA in 0 to 6 months developed by the Food and Agriculture Organization (FAO) in 2010 was 0.1%E~0.18%E (58 mg ~ 104 mg/d) and 0.2%E~0.3%E (115mg~173mg / d), respectively. The Chinese Nutrition Society recommends that the appropriate intake (AI, adequate intakes) value of DHA is 100 mg/d and 150mg / d for ARA. The AI of DHA in infants aged 7 to 36 months is 100 mg/d, based on the recommended AI value of FAO and EFSA for linoleic acid at this age, 3.0%E~4.5%E, which is considered sufficient to meet the needs of infants for ARA synthesis, so the AI value of ARA is not recommended.

[55]Koletzko B, Lien E, Agostoni C, et al.The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations.J.Petrinat.Med.2008, 36(1):5-14.

[56] Urwin HJ, Zhang J, Gao Y, et al.Immune factors and fatty acid composition in human milk from river/lake, coastal and inland regions of China.Br.J.Nutr.2013, 109(11):1949-1961

3.4 Bifidobacterium long long subspecies BB536

Bifidobacterium BB536 isolated the intestines of healthy infants in 1969 and has been approved for infant food in Japan and the United States. It is the first affinity human Bifidobacterium strain (HRB) recognized by the US FDA. At the same time, in 2022, it was approved to be included in China's list of edible fungus list.

As of March 2022, more than 220 academic papers were published based on BB536. A number of clinical studies on infants at home and abroad have proved that the strain has good food safety. Studies have proved that BB536 is effective in maintaining intestinal health, improving immunity, and preventing infection.

3 Study purpose and significance

To compare the effects of breast milk oligosaccharide and multinutrient-optimized infant formula and conventional infant formula on the intestinal health of infants under 1 year of age. In comparison with breast-fed infants, the effects of different infant formulas on infant growth and development, cognitive development, immune function, intestinal comfort, crying, and upper respiratory tract infection were explored.

4 research contents

This trial is a multicenter, randomized, controlled, parallel, double-blind, exploratory trial to compare the effects of infant formula, conventional infant formula on infant formula and breast milk in early life.

240 subjects are expected to be required for this trial. From 0 to 28 days after birth, infants who had signed informed consent by a guardian were screened. Guardian according to their own will choose to attend breastfeeding group or artificial feeding group (mother for subjective and objective reasons for exclusive breastfeeding, can choose to join artificial feeding group, the researchers shall not interfere in the decision of the guardian or guide), breast feeding group and artificial feeding group subjects of 80 cases and 160 cases, respectively.160 subjects in the artificial feeding group were randomly assigned to the experimental group and the control group in a ratio of 1:1 ratio. The experimental formula and control formula were used for artificial feeding or mixed feeding. Artificial feeding was conducted no less than 2 times a day at 0 to 4 months and no less than 5 times a day at 4 to 12 months. Infants in the breastfeeding group need to be breastfed or mixed fed, exclusively breastfeeding daily at 0 to 4 months of age (must be fully breastfed 1 day before the visit), and breastfeeding at least once a day at 4 to 12 months of age.

From the perspective of ethical and subject protection, if the infant and guardian

are eligible and willing to breastfeeding, we will assign the subject to the breastfed group, rather than against the wishes of the parent. If the parent / legal guardian is unable to breastfeed exclusively for objective reasons, or clearly and strongly willing not to breast, these infants will be randomized to the trial or the control group. It should be noted that during the decision-making process, the investigator should encourage the parents / guardian to breastfeeding the infant; rather than interfering or guiding the decision-making process to induce the test infant to participate in the artificial feeding group.

Visit 1 will be performed on the 28th day after birth and the intervention period will last 332 days (slightly changed by clinic visit time) consisting of 3 visits: Visit 2 (90 ± 3 days after birth), Visit 3 (180 ± 5 days after birth), and Visit 4 (360 ± 7 days after birth). Subjects in each group will be enrolled 1:1 and based on an expected completion rate of 80%, 64 subjects are expected to complete the trial. Participants who withdrew from the study before Visit 4, failed in three contacts, met the withdrawal criteria, seriously violated the trial protocol, or met other withdrawal criteria were excluded by the investigators.

In addition, indicators and incidence of adverse events (e. g. eczema) will be recorded and compared during the study. For the purposes of this trial, adverse events were defined as any adverse and unexpected signs (including other abnormal laboratory results beyond the study plan), symptoms, disease related to participation in the clinical trial, and the disease related to the trial product.

5 subject investigated

Infants born 0 to 28 days after birth are screened and who meet all inclusion criteria and do not meet any exclusion criteria will be assigned to the breastfeeding group or randomized to the trial / control group.

5.1 Inclusion criteria

5.1.1 Breastfeeding group

Subjects in the breastfeeding group included in this study must meet all of the following criteria:

- The 0 ~ 28 days after birth;
- Single-child birth;
- The gestational age was from 37 to 42 weeks;
- Birth weight is 2500 ~ 4000 g;

- Parents or guardians agree to exclude the infant from participating in other interventional clinical studies during this study;
- Parents or guardians agreed to feed the infants according to the trial protocol.

5.1.2 Artificial feeding group

Subjects in the artificial feeding group included in this study must meet all of the following criteria:

- The 0 ~ 28 days after birth;
- Single-child birth;
- The gestational age was from 37 to 42 weeks;
- Birth weight is 2500 ~ 4000 g;
- Parents or guardians agree to exclude the infant from participating in other interventional clinical studies during this study;
- Parents or guardians agreed to feed the baby according to the trial protocol;
- No exclusive breastfeeding mothers for subjective and objective reasons.

5.2 Exclusion criteria

Subjects will not be included in this trial if they meet any of the following criteria:

- Artificial assisted reproduction;
- The mother suffers from diseases that may endanger the intrauterine growth;
- The mother had gestational diabetes or severe metabolic disease, chronic disease at the time of pregnancy
- Having congenital malformations and genetic, chronic, and congenital diseases that may interfere with the investigation;
- IgE mediated milk protein allergy or factors that increase the risk of milk protein allergy;
- Having an acute infection or gastroenteritis;
- Functional gastrointestinal diseases, such as gastroparesis;
- Is participating in other clinical trials;

• The investigator was unable to determine the parents' willingness or ability to comply with the protocol requirements.

5.3 Exit standard

• Ineligible (ignored during screening, occurred during the study or found by review);

• Serious protocol deviation occurs;

• Serious non to protocol or test requirements;

• Need to terminate the designated formula due to adverse events or cause failure to continue following the trial procedures;

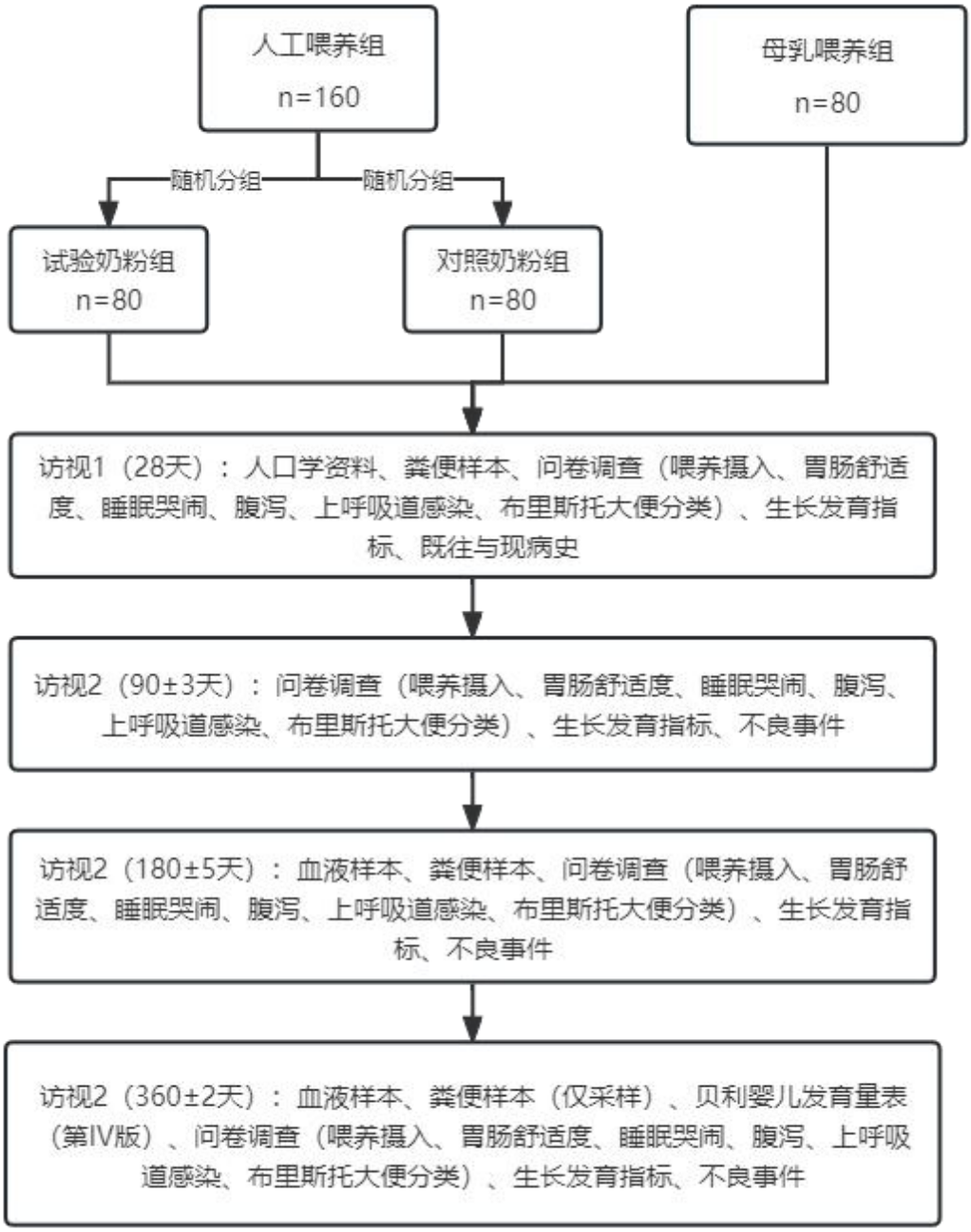
• Need to discontinue the designated formula or cause the inability to follow the trial procedures due to disease progression;

• Withdraw informed consent or remove informed consent;

• Misvisit.

6 research design

6.1 Test flow chart



6.2 recruit

The investigator will identify suitable infants, contact their parents or guardians by telephone and otherwise, and make an appointment for informed consent and subject screening.

6.3 informed consent

The subject's guardian must personally sign the informed consent form and date before conducting any trial process. If the version is updated, contact the guardian to sign the latest version of the informed consent.

A written version of the informed consent was provided to the guardian of the subject, and the investigator should explain the following: the nature of the trial; the content of the subject; the requirements of the protocol; known side effects and any risks involved in participation. The guardian should also be clearly informed that the baby can be withdrawn from the study at any time for any reason, without affecting the baby's medical treatment, legal rights, and no obligation to provide a reason for the withdrawal.

The guardian of the subject was allowed enough time to consider the relevant information and the opportunity to ask the researcher, their GP or other independent party to decide whether to enroll the infant in the trial. The subject's guardian will then sign and date the informed consent form, followed by the investigator giving the informed consent. The investigator who gave the informed consent must have the appropriate qualifications and experience and must be authorized by the principal investigator at each institution. A copy of the signed informed consent form will be given to the subject's guardian for retention and the original remains at the study institution.

6.4 dressing by screening

The investigator will ask the guardian, investigate the basic information of the subject infant, fill in the screening record and the Subject Screening and Enrollment Registration Form, and the infant will be included in the study after meeting the inclusion and exclusion criteria. If the guardian chooses to exclusively breastfeed the infant, the infant will be assigned to the breastfeeding group; if due chooses artificial or mixed feeding, the infant will be randomized to the trial or control group.

6.5 randomization

6.5.1 Random group table

Randtrial block randomization. The randomization tables were generated simulated on a computer by an unblinded statistician using the SAS software according to the random number table. Trial or control milk powder were coded according to the randomization table. Each center will compete for entry. In clinical operation, the authorized investigator or the research assistant will distribute the test or control milk powder according to Powder No. Any deviations should be truthfully

recorded for evaluation before data analysis.

6.5.2 Sample size and calculation basis

[57]The study enrolled 88 test group, 87 control infants, 34 breastfed infants with 1g / L 2′ / FL and 0.5 g / L LNnT to formula-fed infants, control formula-fed infants and breast-fed infants. All the fecal samples available at 3 months of age were 58 in the test group, 65 in the control group, and 34 in the breastfeeding group. They evaluated the microbiota using 16S rRNA gene sequencing and metagenomics, and found that the microbiota composition of the test group was different from that of the control group and closer to that of the breastfeeding group. In this study, the experimental group, control group and breastfeeding group are planned to include 80 subjects, calculated by the shedding rate (failure rate) of 20%. The effective sample size of each group is expected to be 64 cases, which can meet the study needs.

6.6 Interview

6.6.1 Visit 1

Visit 1 will be performed on the 28th day of life and the investigator will obtain the following information:

· Mode of delivery;

· Pregnancy-related complications;

· Parents' physical condition (height, weight, medical history);

· Parents' living habits (smoking history, drinking history, exercise habits);

· Parents' social and economic background (education level, employment status);

· Growth and development parameters (height, head circumference, chest circumference, body weight);

• Stool samples (16S rRNA, SCFAs, sIgA);

· Gastrointestinal comfort survey and questionnaire;

· Questionnaire on baby crying condition;

· Questionnaire on infant diarrhea;

· Questionnaire on upper respiratory tract infection in infants;

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[57]Steenhout P , Sperisen P , Martin F , et al.Term Infant Formula Supplemented with Human Milk Oligosaccharides (2′ Fucosyllactose and Lacto‐N‐neotetraose) Shifts Stool Microbiota and Metabolic Signatures Closer to that of Breastfed Infants[J].The FASEB Journal, 2016, 30.

· The Bristol Stool Classification Questionnaire;

· Past medical history and current medical history.

6.6.2 Visit 2

Visit 2 will be conducted on Birth Day 90 with a window of 3 days and the investigator will measure and obtain the following information:

· Growth and development parameters (height, head circumference, chest circumference, body weight);

· Gastrointestinal comfort survey and questionnaire;

· Questionnaire on baby crying condition;

· Questionnaire on infant diarrhea;

· Questionnaire on upper respiratory tract infection in infants;

· The Bristol Stool Classification Questionnaire;

· Adverse events and serious adverse events.

6.6.3 Visit 3

Visit 3 will be conducted on Post Birth Day 180 with a window of 5 days and the investigator will measure and obtain the following information:

· Growth and development parameters (height, head circumference, chest circumference, body weight);

- Blood samples (hsCRP, analysis of lymphocyte subsets, amino acids, osteocalcin, alkaline phosphatase, albumin);
- Stool samples (16S rRNA, SCFAs, sIgA);

· Gastrointestinal comfort survey and questionnaire;

· Questionnaire on baby crying condition;

· Questionnaire on infant diarrhea;

· Questionnaire on upper respiratory tract infection in infants;

· The Bristol Stool Classification Questionnaire;

· Adverse events and serious adverse events.

6.6.4 Visit 4

Visit 4 will be performed on Post Birth Day 360 with a window of 7 days and the investigator will measure and obtain the following information:

· Growth and development parameters (height, head circumference, chest circumference, body weight);

· Bailey Infant Development Scale (Version IV);

· Blood samples (hsCRP, lymphocyte subsets, amino acids, osteocalcin, alkaline phosphatase, albumin);

• Stool samples (16S rRNA, SCFAs, sIgA);

· Gastrointestinal comfort survey and questionnaire;

· Questionnaire on baby crying condition;

· Questionnaire on infant diarrhea;

· Questionnaire on upper respiratory tract infection in infants;

· The Bristol Stool Classification Questionnaire;

· Adverse events and serious adverse events.

6.7 Subjects stopped / withdrew from the trial

The guardian of the subject has the right to withdraw the infant from the trial at any time. In addition, the investigator may discontinue the subject at any time if the investigator considers it necessary for reasons including:

• Ineligible (ignored during screening, occurred during the study or found by review);

• Serious protocol deviation occurs;

• Serious non to protocol or test requirements;

• Termination of the designated formula or failure to follow the trial procedures due to an adverse event;

• Need to discontinue the designated formula or cause the inability to follow the trial procedures due to disease progression;

• Withdraw informed consent or remove informed consent;

• Misvisit.

If a subject withdraws from the trial due to an adverse event, the investigator will schedule a follow-up visit or telephone visit until the adverse event is resolved or is stable. Data collected before withdrawal will be used for analysis. If a subject withdraws from the trial, the subject will not be replaced. The reason for the withdrawal will be documented in the case report form.

7 Research products

7.1 burden

(1) Ingredients list of test milk powder

The test milk powder was produced by Feihe (Jilin) Dairy Co., LTD. Three formulations were used for infants aged 0-3 months, 3-6 months and 6-12 months respectively. All products are produced in strict accordance with Good Production Practice (GMP), and all indicators meet the requirements of relevant standards and regulations. Compared with the control milk powder, 2 ′ -fucinose based lactose, lactose-N-new tetrosaccharide, bifidobacterium longum BB536 are the added ingredients unique to the test milk powder, and the ingredients are as follows:

0 to 3 months old infants: raw milk, lactose, edible plant blend oil (1,3-linoleic acid 2-palmitate triglyceride, sunflower oil, coconut oil, flaxseed oil), desalting whey powder, oligo galactose, whey protein powder, whey protein powder, 2'-fucoose based lactose, lactose-N-new four sugar, phospholipids, vitamin A, vitamin D, vitamin E, vitamin K, vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, Niacin, folic acid, pantothenate, vitamin C, biotin, choline, sodium citrate, potassium chloride, copper sulfate, magnesium sulfate, iron pyrophosphate, zinc sulfate, manganese sulfate, calcium carbonate, calcium hydrogen phosphate, potassium iodate, sodium selenite, innositol, taurine, docosahexaenoic acid (DHA), arachidonic acid (ARA), lutein, nucleotide, disodium disdium 5'-monophosphate, 5 ′, disodium), Bb-12, Bifidobacterium longum B B536.

3-6 months old infants: raw milk, lactose, edible plant blend oil (1,3-linoleic acid 2-palmitate triglyceride, sunflower oil, coconut oil, flaxseed oil), oligo galactose, whey protein powder, desalting whey powder, whey protein powder, 2'-fucoidan based lactose, lactose-N-new four sugar, phospholipids, vitamin A, vitamin D, vitamin E, vitamin K, vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, Niacin, folic acid, pantothenic acid, vitamin C, biotin, biotin, choline, sodium citrate, potassium chloride, copper sulfate, magnesium sulfate, iron pyrophosphate, zinc sulfate, manganese

sulfate, calcium carbonate, calcium hydrogen phosphate, potassium iodiite, sodium selenite, inositol, hexanotide (DHA), arachidonic acid (ARA), lutein, nucleotide, disodium, 5′-monomonophosphate, disodium), lactoferrin, Bb-12, Bifidobacterium longum B B536.

6-12 months old infants: raw milk, lactose, edible plant blend oil (1,3-linoleic acid 2-palmitate triglyceride, sunflower oil, coconut oil, flaxseed oil), oligo galactose, whey protein powder, milk powder, whey protein powder, 2'-fucose lactose, lactose-N-new tetrosaccharide, phospholipids, vitamin A, vitamin D, vitamin E, vitamin K, vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, Niacin, folic acid, pantothenic acid, vitamin C, biotin, biotin, choline, sodium citrate, potassium chloride, copper sulfate, magnesium sulfate, iron pyrophosphate, zinc sulfate, manganese sulfate, calcium carbonate, calcium hydrogen phosphate, potassium iodiite, sodium selenite, inositol, hexanotide (DHA), arachidonic acid (ARA), lutein, nucleotide, disodium, 5′-monomonophosphate, disodium), lactoferrin, Bb-12, Bifidobacterium longum B B536.

(2) Control with the ingredients list of milk powder

The control milk powder is commercially available infant formula, with a total of two formulas, which are suitable for infants aged 0-6 months and 6-12 months respectively. All the indicators of the products meet the requirements of the relevant standards and regulations. The list of ingredients is as follows:

0 to 6 months old baby: raw milk, desalting whey powder, edible plant blend oil (1,3-linoleic acid 2-palmitate triglyceride, sunflower oil, coconut oil, linseed oil), lactose, oligo galactose, walnut oil, whey protein powder, whey protein powder, vitamin A, vitamin D, vitamin E, vitamin K, vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, Niacin, folic acid, pantothenate, vitamin C, biotin, choline, sodium citrate, potassium chloride, copper sulfate, magnesium sulfate, iron pyrophosphate, zinc sulfate, manganese sulfate, calcium carbonate, calcium hydrogen phosphate, potassium iodate, sodium selenite, innositol, taurine, levosnitine, dococarpohexenoic acid (DHA), arachidonic acid (ARA), lutein, lutein, nucleotide (5′-cytitilate, 5′, 5′ monophosphate, 5′, 5′-inolate), bifidobacterium Bb-12.

6-12 months of older infants: raw milk, lactose, desalting whey powder, edible plant blend oil (1,3-linoleic acid 2-palmitate glyceride, sunflower oil, coconut oil, linseed oil), milk powder, oligomic galactose, whey protein powder, whey protein powder, walnut oil, vitamin A, vitamin D, vitamin E, vitamin K, vitamin B_1, vitamin

B_2, vitamin B_6, vitamin B_{12}, Niacin, folic acid, pantothenate, vitamin C, biotin, choline, sodium citrate, potassium chloride, copper sulfate, magnesium sulfate, iron pyrophosphate, zinc sulfate, manganese sulfate, calcium carbonate, calcium hydrogen phosphate, potassium iodate, sodium selenite, innositol, taurine, levosnitine, dococarpohexenoic acid (DHA), arachidonic acid (ARA), lutein, lutein, nucleotide (5′-cytitilate, 5′, 5′ monophosphate, 5′, 5′-inolate), bifidobacterium Bb-12.

7.2 Nutritional composition table

（1） Nutrition composition list of test milk powder

Table 1. Nutrient composition list of test samples at 0-3 months of age

project	unit	Every 100 kJ	Every 100g
energy	**kJ**	**100**	**2097**
protein	**g**	**0.60**	**12.5**
fat	**g**	**1.24**	**26.0**
linoleic acid	g	0.20	4.1
α-linolenic acid	mg	20	410
carbohydrate	**g**	**2.5**	**52.3**
vitamin			
vitamin A	μg RE	17	360
vitamin D	μg	0.62	13.0
vitamin E	mg α-TE	0.29	6.00
vitamin K_1	μg	2.86	60.0
vitamin B_1	μg	31	650
vitamin B_2	μg	24	500
vitamin B_6	μg	19.1	400
vitamin B_{12}	μg	0.072	1.50
niacin	μg	196	4100
folic acid	μg	4.3	90
pantothenic acid	μg	145	3050
vitamin C	mg	3.3	70.0
biotin	μg	0.76	16.0
bilineurine	mg	10.3	215.0
mineral substance			
sodium	mg	8	175
potassium	mg	18	385
copper	μg	16.7	350
magnesium	mg	1.5	32
iron	mg	0.24	5.0

zinc	mg	0.21	4.50
manganese	μg	1.91	40
calcium	mg	17	350
phosphorus	mg	10	220
iodine	μg	5.1	106.0
chlorine	mg	13	280
Se	μg	0.91	19.0
Available ingredients			
inose	mg	1.9	40.0
taurine	mg	1.8	38.0
Docosahexaenoic acid (DHA)	mg	4.6	97.0
Eicosapentetraenoic acid (ARA)	mg	7.9	164.9
The oligomeric galactose	g	0.14	3.0
2′-fucoose-based lactose	g	0.037	0.78
Lactose-N-neotetrasaccharide	g	0.019	0.39
1,3-dioleic acid 2-palmitate triglyceride	g	0.36	7.5
lutein	μg	3.8	80
ribotide	mg	1.4	30.0
lactoferrin	mg	23.8	500.0

Table 2 Nutrient composition list of 3-6 month old test samples

project	unit	Every 100 kJ	Every 100g
energy	**kJ**	**100**	**2097**
protein	**g**	**0.45**	**9.5**
fat	**g**	**1.24**	**26.0**
linoleic acid	g	0.20	4.1
α-linolenic acid	mg	20	410
carbohydrate	**g**	**2.6**	**55.3**
vitamin			
vitamin A	μg RE	17	360
vitamin D	μg	0.62	13.0
vitamin E	mg α-TE	0.29	6.00
vitamin K_1	μg	2.86	60.0
vitamin B_1	μg	31	650
vitamin B_2	μg	24	500
vitamin B_6	μg	19.1	400
vitamin B_{12}	μg	0.072	1.50
niacin	μg	196	4100
folic acid	μg	4.3	90
pantothenic acid	μg	145	3050

vitamin C	mg	3.3	70.0
biotin	μg	0.76	16.0
bilineurine	mg	10.3	215.0
mineral substance			
sodium	mg	8	175
potassium	mg	18	385
copper	μg	16.7	350
magnesium	mg	1.5	32
iron	mg	0.24	5.0
zinc	mg	0.21	4.50
manganese	μg	1.91	40
calcium	mg	17	350
phosphorus	mg	10	220
iodine	μg	5.1	106.0
chlorine	mg	13	280
Se	μg	0.91	19.0
Available ingredients			
inose	mg	1.9	40.0
taurine	mg	1.8	38.0
L-carnitine	mg	0.4	9.0
Docosahexaenoic acid (DHA)	mg	4.6	97.0
Eicosapentetraenoic acid (ARA)	mg	7.9	164.9
The oligomeric galactose	g	0.14	3.0
2′-fucoose-based lactose	g	0.037	0.78
Lactose-N-neotetrasaccharide	g	0.019	0.39
1,3-dioleic acid 2-palmitate triglyceride	g	0.36	7.5
lutein	μg	3.8	80
ribotide	mg	1.4	30.0
lactoferrin	mg	23.8	500.0
Casein phosphopeptides	mg	6.2	130.0

Table 3 6-12 months of age test samples

project	**unit**	**Every 100kJ**	**Every 100g**
energy	**kJ**	**100**	**2012**
protein	**g**	**0.57**	**11.5**
fat	**g**	**1.07**	**21.5**
linoleic acid	g	0.14	2.8
α-linolenic acid	mg	14	280
carbohydrate	**g**	**2.9**	**58.3**

vitamin			
vitamin A	μg RE	22	450
vitamin D	μg	0.60	12.0
vitamin E	mg α-TE	0.24	4.90
vitamin K_1	μg	2.24	45.0
vitamin B_1	μg	30	600
vitamin B_2	μg	38	760
vitamin B_6	μg	14.9	300
vitamin B_{12}	μg	0.060	1.20
niacin	μg	194	3900
folic acid	μg	3.0	60
pantothenic acid	μg	204	4100
vitamin C	mg	3.3	66.0
biotin	μg	0.60	12.0
bilineurine	mg	9.9	200.0
mineral substance			
sodium	mg	7	135
potassium	mg	21	420
copper	μg	11.9	240
magnesium	mg	1.6	33
iron	mg	0.30	6.0
zinc	mg	0.20	4.00
manganese	μg	1.89	38
calcium	mg	22	450
phosphorus	mg	13	270
iodine	μg	4.5	90.0
chlorine	mg	14	280
Se	μg	0.60	12.0
Available ingredients			
inose	mg	2.0	40.0
taurine	mg	1.9	38.0
L-carnitine	mg	0.5	10.0
Docosahexaenoic acid (DHA)	mg	4.7	95.0
Eicosapentetraenoic acid (ARA)	mg	8.0	161.5
The oligomeric galactose	g	0.15	3.0
2′-fucoose-based lactose	g	0.024	0.48
Lactose-N-neotetrasaccharide	g	0.010	0.21
1,3-dioleic acid 2-palmitate triglyceride	g	0.25	5.0

lutein	μg	10.4	210
ribotide	mg	1.5	30.0
lactoferrin	mg	24.9	500.0
Casein phosphopeptides	mg	8.3	167.0

（2） Nutrient composition table of the control samples

Table Nutrient composition list of control samples from 4 0 to 6 months of age

project	**unit**	**Every 100kJ**	**Every 100g**
energy	**kJ**	**100**	**2173**
protein	**g**	**0.48**	**10.5**
fat	**g**	**1.25**	**27.2**
linoleic acid	g	0.17	3.8
α-linolenic acid	mg	19	423
carbohydrate	**g**	**2.6**	**57.3**
vitamin			
vitamin A	μg RE	17	360
vitamin D	μg	0.60	13.0
vitamin E	mg α-TE	0.28	6.00
vitamin K_1	μg	2.58	56.0
vitamin B_1	μg	29	623
vitamin B_2	μg	28	600
vitamin B_6	μg	18.4	400
vitamin B_{12}	μg	0.069	1.50
niacin	μg	189	4100
folic acid	μg	3.3	72
pantothenic acid	μg	138	3000
vitamin C	mg	3.3	72.0
biotin	μg	0.74	16.0
bilineurine	mg	9.9	215.0
mineral substance			
sodium	mg	8	175
potassium	mg	18	400
copper	μg	16.6	360
magnesium	mg	1.4	31
iron	mg	0.21	4.5
zinc	mg	0.18	4.00
manganese	μg	1.98	43
calcium	mg	17	360
phosphorus	mg	10	210

iodine	μg	4.9	106.0
chlorine	mg	14	297
Se	μg	0.87	19.0
Available ingredients			
inose	mg	1.8	40.0
taurine	mg	1.7	38.0
L-carnitine	mg	0.4	9.0
Docosahexaenoic acid (DHA)	mg	4.5	97.0
Eicosapentetraenoic acid (ARA)	mg	7.6	164.9
The oligomeric galactose	g	0.08	1.7
1,3-dioleic acid 2-palmitate triglyceride	g	0.27	5.8
lutein	μg	3.0	66
ribotide	mg	1.4	30.0

Table 5 Nutrient composition list of control samples from 6-to 12 month old

project	**unit**	**Every 100 kJ**	**Every 100g**
energy	**kJ**	**100**	**2058**
protein	**g**	**0.56**	**11.5**
fat	**g**	**1.07**	**22.0**
linoleic acid	g	0.14	2.8
α-linolenic acid	mg	14	280
carbohydrate	**g**	**3.0**	**61.0**
vitamin			
vitamin A	μg RE	22	450
vitamin D	μg	0.58	12.0
vitamin E	mg α-TE	0.22	4.50
vitamin K_1	μg	1.75	36.0
vitamin B_1	μg	30	610
vitamin B_2	μg	34	700
vitamin B_6	μg	14.6	300
vitamin B_{12}	μg	0.058	1.20
niacin	μg	190	3900
folic acid	μg	2.9	60
pantothenic acid	μg	155	3200
vitamin C	mg	3.3	67.0
biotin	μg	0.78	16.0
bilineurine	mg	9.7	200.0
mineral substance			

sodium	mg	7	135
potassium	mg	21	430
copper	μg	11.7	240
magnesium	mg	1.6	33
iron	mg	0.29	6.0
zinc	mg	0.19	4.00
manganese	μg	1.85	38
calcium	mg	22	450
phosphorus	mg	13	270
iodine	μg	4.4	90.0
chlorine	mg	12	250
Se	μg	0.58	12.0
Available ingredients			
inose	mg	1.2	25.0
taurine	mg	1.8	38.0
L-carnitine	mg	0.4	9.0
Docosahexaenoic acid (DHA)	mg	4.6	95.0
Eicosapentetraenoic acid (ARA)	mg	7.8	161.5
The oligomeric galactose	g	0.07	1.4
1,3-dioleic acid 2-palmitate triglyceride	g	0.18	3.8
lutein	μg	10.2	210
ribotide	mg	1.5	30.0

7.3 Sample management

All trial milk powder for the study will be provided to the subjects free of charge. The trial and control powder were stored in the central warehouse and distributed by the investigator by subject number and trial protocol.

8 Outcome indicators

8.1 Main end point

Intestinal indicators (16S rRNA, SCFAs).

8.2 Secondary end point

Inflammation index: serum hsCRP; nutritional index: serum albumin; growth and development index: height, weight, head circumference, chest circumference; immune index: fecal sIgA, blood lymphocyte subgroup; bone development index: osteocalcin, alkaline phosphatase; tolerance index: survey of infant gastrointestinal comfort, infant sleep crying, infant diarrhea and upper respiratory tract infection,

infant stool Bristol stool classification; cognitive development index: Bailey Infant Development Scale (IV edition), serum amino acids.

8.3 Safety evaluation index

Adverse events, serious adverse events.

9 Data statistics method

9.1 Data analysis software

Demographic, safety and efficacy data were analyzed using the SAS (version 9.4) software.

9.2 General data analysis

Statistical description is mainly used, and statistical inference is used for reference only. Quantitative data were expressed by n (number of subjects with non-missing values), mean, median, standard deviation, maximum value, and minimum value, and qualitative data were expressed by frequency and percentage.

The missing values in the statistical analysis involved in this statistical analysis plan will not be inserted. Outliers are determined in a unified discussion before the database lock library.

9.3 Analyze the crowd

The Statistical analysis population data sets were defined as follows:

Screening Set (Screening Set): includes all subjects who signed the informed consent and participated in trial screening.

Full Analysis Set (Full Analysis Set, FAS): All subjects who participated in the trial and completed at least Visit 1. The FAS set is the main population for the efficacy analysis.

Compliance Set (Per-Protocol set, PPS): is a subset of the full analysis set containing subjects who completed all visits to the trial and had no major protocol deviations during the trial. The PPS set is a secondary population for the efficacy analysis.

Safety Analysis Set (Safety Set, SS): The Safety Analysis Set includes all subjects who participated and received test samples with records of safety indicators.

9.4 Subject completion status

To clarify the implementation of the clinical trial and the status of the clinical

trial analysis set, a clear summary of the subjects from the completion of the clinical trial, including the following:

（1） Subject Clinical Trial Completion Form, including

- Number of screened subjects;
- Number of unlisted subjects;
- Number of randomized subjects;
- Number and percentage of subjects who have completed the clinical trial;
- Number and percentage of subjects withdrawing early from the clinical trial;
- And to distinguish the number and percentage of subjects withdrawing early from the clinical trial for different reasons.

（2） Number and percentages of the different analysis sets.

（3） Protocol deviation summary.

（4） Subject completion in the clinical trial and the analysis set are provided in a list form.

9.5 Demographic data

Subject demographic data will be summarized according to the full analysis set. The summary of demographic data should include the following: (1) age (age year) - - the difference between the date of signed informed consent and the date of birth / 365.25; (2) gender- -male / female; (3) ethnic group- -Han / other.

9.6 adverse event

All reported adverse event names and terminologies need to be coded in the latest version of the MedDRA. For all adverse event tables, subjects were counted only once in each preferred term (Preferred Term, PT) and systemic organoids (System Organ Class, SOC). Unless specifically defined, adverse events were ranked in descending order of the preferred term frequency in each system organ class. If several preferred terms in the same system organclass have the same frequency, then in the literal order of preferred terms.

All adverse events will be summarized by SOC, PT and CTCAE criteria, including

- Number of subjects, percentage of adverse events, and number of adverse events
- Number and percentage of subjects with serious adverse events and number of adverse events
- Number of subjects with early withdrawal due to adverse events and number of adverse events

All adverse events will be displayed as lists.

9.7 efficiency analysis

9.7.1 Main endpoint indicators

Primary endpoint index: Changes in the intestinal flora of infants compared between the test group and the control group.

9.7.2 statistical analysis technique

16 The lowest ranked taxon O TUs was obtained from sequencing data for further analysis.

（1） Analysis of the bacterial species diversity profile

- The diluability curve (Rarefaction curve) is used to analyze whether the existing OTU data can reflect the diversity of the sample flora, and the sequencing depth is used to evaluate whether the sequencing data amount of the sample is reasonable. Random sampling of sequences is used to construct the number of sequences drawn and the number of OTU they can represent. When the curve is flat, it indicates that the amount of sequencing data is reasonable.
- Species composition analysis was conducted, and according to the species information and abundance information of O TUs, the appropriate taxonomic level (species <genus <family <order <class <phyla) was selected to draw the colony columns of different groups of samples.
- The Alpha Diversity (Alpha diversity) index was used to quantify the bacterial community characteristics of the samples, as described by the S obs, C hao, Ace, Shannon, and S impson index. The S obs Index, Chao, index and Ace index can estimate the number of species in the sample, and the Shannon index and S impson index reflect the species richness and species uniformity of the sample.

（2） Analysis of microflora composition differences among samples

- Differences in species composition between different groups were analyzed using W ilcoxon (comparing two groups) and K ruskal-Wallis (three groups and above) to compare differences in Alpha diversity index between groups, if $P < 0.05$.

- Beta diversity (Beta diversity) analysis was used to use the species composition or evolutionary relationship between species and abundance information to calculate the inter-sample distance, and the similarity or difference of community composition between different grouped samples was explored. The results are shown in Heatmap diagram.
- Using p rincipal component analysis (p rincipal component analysis, PCA) to assess whether infant samples from different groups can be distinguished. When the sample composition is more similar, the closer the distance reflected in the PCA plot is. Samples from different backgrounds may exhibit a distribution of dispersion and aggregation.
- Using W ilcoxon and K ruskal-Wallis tests by LEfSe software to identify significantly different species between groups and visually show the distribution of different species between groups at each taxonomic level.

（3） Association analysis of environmental factors

- Correlation analysis was used to explore the associations between infant gut microbiota and environmental factors (sex, birth weight, etc.) in different groups. Correlation coefficients were calculated using P earson and S pearman when samples cannot satisfy a continuous normal distribution and a linear relationship. The results are presented as the Heatmap plots.

10 Management plan for common / serious adverse events

10.1 Common adverse events / serious adverse events

The test and control milk powder contain lactose and milk protein, and some infants may have symptoms of lactose intolerance or milk protein allergy. Lactose intolerant populations were excluded at screening to maximize potential hazards. Trial and control milk powder generally do not result in the occurrence of serious adverse events.

10.2 Management of the adverse events

An adverse event is an adverse event after a patient or a clinical study subject receives an investigational product that does not necessarily be causally related to the trial. Adverse events are laboratory findings of disease, signs or symptoms, and / or abnormalities during the course of the study. Adverse events include subjects in contacting the investigator or their personal physician, being examined or given medical guidance. This may or may not cause the subject's withdrawal from the study. Adverse events are generally classified as serious or not serious.

All adverse events during the study should be reported and recorded, regardless of whether they are considered serious or not serious and treatment-related. Each case should include the following: subject and date, description of the event, duration, frequency, degree, severity, actions taken, results and sequelae, and relationship to the tested product.

10.3 Management of serious adverse events

Record of serious adverse events (SAEs) requires an independent table, completed by the investigator for each case.

In addition, serious adverse events were reported to the EC by the investigator through the project leader. Such events should be informed immediately by telephone and the SAE form reported within 48 hours.

After an adverse event occurs, if further information examination is required to evaluate the relationship between the adverse event and the test sample, all tests or laboratory results must be attached to the CRF or follow-up records.

10.4 Subject safeguards

Subjects participating in the clinical trial will be purchased clinical trial liability insurance. All the subjects use adverse events or serious adverse events during the clinical trial, and make economic compensation according to the severity of the adverse event.

11 Ethical requirements

11.1 Protection of subject privacy

The privacy of all participants will be protected and subjects will be identified by an identification code.

11.2 informed consent

The investigator receives written informed consent from each subject before each subject performs enrollment screening. The informed consent form will be signed and dated by the investigator and the subject. The informed consent form is in duplicate: the first remains in the investigator folder and the second to the subject. Testing could not be performed on subjects until written informed consent.

11.3 Approval of the ethics committee

The Investigator is to submit the study protocol to the Institutional Review Board (IRB) / Independent Ethics Committee (IEC) for review. Clinical studies could not be performed without written approval from the ethics committee.

Any additions or changes to the future study protocol will require notification of the IRB / IEC. The IRB / IEC should also be notified if any serious adverse event occurs during the clinical study.

11.4 Declaration of Helsinki

This study will be conducted in accordance with the principles and provisions of the Declaration of Helsinki and the subsidiary articles.

12 Quality control and quality assurance

The implementation process of this trial will refer to the trial protocol, the Good Laboratory Practice for Drug Clinical Trial issued by the State Food and Drug Administration and the NHC (2020 edition), and comply with relevant laws and regulations.

The investigator shall strictly follow the protocol; the hospital will manage and quality control the trial as required; an independent clinical trial monitor (CRA) will monitor the quality of the test; the CRA will monitor and report the project weekly.

13 Study termination

If it is clear that the study is be continued early before the completion of the study, the investigator will notify the Ethics committee of the reason in advance. Data not released / not used in the study, the test samples will be destroyed or returned.