Gut microbiota and fecal calprotectin levels in preterm infants with feeding intolerance and necrotizing enterocolitis: An observational study

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Abstract. Preterm infants with a lower gestational age have a higher risk of developing gastrointestinal (GI) pathologies, such as feeding intolerance (FI) and necrotizing enterocolitis (NEC). Infants with a very low birth weight (VLBW) and an extremely low birth weight (ELBW) are prone to pathogenic gut colonization due to prolonged periods of hospitalization in intensive care units (ICUs), maternal or infant antibiotic use, and difficulties with breastfeeding related to marked morphofunctional and immune immaturity. Despite available data on the pathogenetic role of dysbiosis and pathogenic flora in FI and NEC, consistent microbiome-related risk factors and clear diagnostic approaches have not yet been defined, and the search for reliable noninvasive intestinal markers continues. The aim of the present study was to determine whether pathogenic intestinal colonization and elevated fecal calprotectin (FC) levels are associated with FI and NEC in preterm infants with a low birth weight. For this purpose, the present prospective cohort study compared gut microbiota composition and FC levels in preterm infants with and without FI. The present study included preterm infants with a VLBW or ELBW (birth weight, <1,500 g; age >14 days). Infants with FI formed the study group (n=57), and those without GI symptoms served as the controls (n 21). Preterm infants with a VLBW or ELBW and FI (n=57) were found to carry pathogenic flora, including *Klebsiella pneumoniae* (29.8%), *Klebsiella oxytoca* (22.8%), *Enterobacter cloacae* (17.6%), *Enterobacter aerogenes* (15.8%), *Citrobacter koseri* (10.5%), *Acinetobacter baumannii* complex (1.7%) and *Candida albicans* (7.0%). A total of 10 infants (17.5%) subsequently developed NEC, accompanied by an increase in the level of FC. N the whole, the present study found that underweight preterm infants with FI in the ICUs had pathogenic or potentially pathogenic microorganisms in their gut microbiota and were at risk of developing NEC. For preterm infants with a VLBW or ELBW in ICUs, the early identification of pathogenic gut colonization and elevated FC levels may help stratify the risk of FI and NEC and guide targeted preventive strategies.

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Abbreviations: ELBW, extremely low body weight; FC, fecal calprotectin; FI, feeding intolerance; ICUs, intensive care units; NEC, necrotizing enterocolitis; VLBW, very low body weight

Key words: extremely low birth weight, very low birth weight, gut microbiota, intestinal dysbiosis, necrotizing enterocolitis, fecal calprotectin

Introduction

Infants with a very low birth weight (VLBW) of 1,000-1,500 g and an extremely low birth weight (ELBW) of 500-999 g are characterized by the morphological and functional immaturity of organs and systems. Premature babies do not always manage to maintain their intrauterine growth rate after birth. Malnutrition in the early stages of development leads to long-term short stature, delayed organ growth, and a deficit in the number of neurons and dendritic connections, as well as later behavioral disorders (1). In addition to the immaturity of the gastrointestinal (GI) tract, reduced motility and the decreased activity of intestinal enzymes, preterm newborns often present with severe comorbid conditions. These conditions are accompanied by inflammatory processes associated with bronchopulmonary dysplasia, necrotizing enterocolitis (NEC) and intraventricular hemorrhages (2,3). In their study, Chen *et al* (4) identified risks and predicted a pattern of feeding intolerance (FI) in children. These children have a low gestational age, a low birth weight, a history of fetal distress, a history of the use of aminophylline, and an interval of >3 days between bowel movements (4).

In recent years, the intestinal microbiome in newborns has been a focus of neonatology due to its critical role in the proper functioning of the body and neonatal health. Newborns have less diversity in their intestinal flora than adults. Intestinal colonization factors in newborns are closely related to gestational age at birth, the mode of delivery and antibacterial therapy (5-7).

According to Neu and Rushing (8), newborns born vaginally acquire bacteria resembling the vaginal microbiome of their mother, mainly *Lactobacillus* and *Prevotella*. On the contrary, newborns born by cesarean section acquire bacteria similar to the skin microbiome of their mother, primarily *Staphylococcus aureus* (8).

Pace *et al* (9) demonstrated the changes in the vaginal microbiome with gestational age. The relative content of *Lactobacillus spp*. in the mother's vaginal microflora increases towards full-term pregnancy. Thus, *Lactobacillus spp*. predominates and determines the spectrum of microorganisms colonizing the newborn during its passage through the birth canal (9).

Factors, such as antibacterial therapy, mechanical ventilation, delayed breastfeeding and low enteral feeding volumes delay the establishment of a normal gut microbiota in infants born prior to 28 weeks of gestation. The resulting prevalence of *Escherichia coli, Klebsiella, Staphylococcus, Propionibacterium* and *Corynebacterium* in premature babies has long-term consequences for their health, including an increased susceptibility to developing NEC (10,11). Normal intestinal biocenosis prevents the development of opportunistic microflora, producing vitamins B, K, and D, and promoting the absorption of iron and calcium (12).

According to the study by Yao *et al* (13), the intestines of preterm infants are colonized with hospital-acquired flora due to their prolonged periods of hospitalization in intensive care units (ICUs) and the long-term use of antibiotics. They emphasized that gut colonization in preterm infants differs significantly from that in full-term infants (13). The inverse association between age at NEC diagnosis and gestational age at birth reflects the accumulation of risk factors, such as dysbiosis and developmental features of the intestinal immune system (14).

FI in preterm infants is directly associated with changes in the gut microbiota. Children with FI have a lower microbial diversity, fewer beneficial bacteria and increased numbers of pathogenic bacteria (15).

A recent literature review demonstrated that intestinal dysbiosis can cause acute illnesses in preterm infants, including NEC, sepsis, bronchopulmonary dysplasia and retinopathy of prematurity, and can affect their neuropsychological development and somatic growth (16).

Altered gut microbiota and its metabolites directly affect nutrient absorption, gut development, inflammation and hormonal signaling, thereby affecting growth, and promoting the development of metabolic disorders in preterm infants. This supports the connection between pathologic gut colonization and the development of bacteremia in preterm infants in ICUs (17). These data highlight the key role of the gut microbiota in the development of inflammatory complications, including NEC, in preterm infants.

The pathogenesis of NEC begins with an inflammatory process that damages the intestinal mucosa colonized by microflora, the immature intestinal epithelium and the intestinal circulation, leading to an excessive inflammatory response (18).

The high incidence and mortality rates associated with FI and NEC among preterm infants (19) emphasize the need for the early identification of risk groups and the development of effective methods for their prevention and early diagnosis. Previous studies have highlighted the role of the gut microbiota in the pathogenesis of these conditions, with a particular focus on *Enterobacter, Klebsiella, Citrobacter* and *Acinetobacter* Gram-negative bacteria (10,19). These microorganisms, often nosocomial pathogens, can colonize the intestine in humans with immature immunity and impaired barrier function, contributing to the development of systemic infections and the exacerbation of inflammatory processes (20). The clinical consequences of a disrupted intestinal microbiome in preterm infants are of particular interest. Prospects for microbiome diagnostics and interventions to improve the health of preterm infants have been discussed (21,22).

Mai *et al* (23) reported increases in Proteobacteria (by 34%) and decreases in Firmicutes (by 32%) within 72 h prior to the onset of the clinical symptoms of NEC. They suggested that such a change in microbiota may represent a pattern of the manifestation of NEC (23).

Almost all preterm infants are prescribed initial antibiotic therapy. It reduces overall microbial diversity and depletes beneficial microorganisms, such as *Bifidobacteriaceae*, which are early colonizers of the intestine. *Bifidobacteriaceae* efficiently ferment breast milk oligosaccharides, which serve as substrates for the synthesis of short-chain fatty acids, an important nutrient source in the intestine. The depletion of protective microorganisms disrupts microbial homeostasis and increases the proportion of pathogenic enterobacteria, such as *Enterobacteriaceae*, which are often associated with antimicrobial resistance and the risk of developing sepsis and NEC (24,25). The high levels of antibiotic resistance and clinical significance of pathogenic microorganisms urge us to understand the factors that contribute to their growth and pathogenicity, to improve the prevention and treatment of NEC and FI.

Both qualitative and quantitative assessments of gut microbiota are critical for understanding its role in FI and NEC. The quantification of bacterial colonization is usually performed in 'colony-forming units per gram' (CFU/g). This approach, based on standard seeding methods followed by colony counting, is considered reliable for identifying and quantifying bacterial cultures in the feces of newborns (26,27).

A search is currently underway for a simple, rapid, reliable, sensitive and safe diagnostic tools which can be used to detect and monitor intestinal inflammation in neonates. Fecal calprotectin (FC) analysis is considered a promising non-invasive marker of inflammation (28). When measured in feces, calprotectin is markedly associated with the neutrophil infiltration of the intestinal mucosal surface and within the gut lumen, and is a hallmark of digestive inflammatory pathology (29,30). The interpretation of FC levels in neonates, particularly preterm infants, should consider age-dependent factors and the degree of intestinal maturity. Some clinical

studies have demonstrated that FC levels can vary significantly during the first weeks of life, including physiologically high values, particularly at 3-78 days (31,32). Such values may be associated not only with an inflammatory reaction, but also with the processes of primary colonization of the intestine by opportunistic microflora (31,32).

There is ample evidence of higher neonatal FC levels in full-term and premature newborns compared to reference values in adults (<50 μg/g) and older children. FC levels can reach 600 mcg/g in clinically healthy newborns and even more in preterm infants, with high interindividual variability. However, FC levels tend to decrease as the intestines adapt postnatally. Notably, the method of feeding (breast or formula) does not significantly affect FC levels (33-36).

Cekovic *et al* (37) reported a positive correlation between high FC levels and intrauterine growth retardation, a prominent arterial duct, intolerance to enteral feeding and the prolonged use of broad-spectrum antibiotics in the postnatal period (37).

Preterm infants with a VLBW of ELBW, particularly those in neonatal ICUs, are exposed to invasive procedures, antibacterial therapy and hospital microflora, which can prevent the development of normal gut microbiota and increase the vulnerability of the intestine to inflammatory and infectious complications. In this population, the characteristics of the gut microbiota and intestinal inflammation require targeted analyses as potential key factors in the pathogenesis of FI and NEC.

The present study used FC as a marker of intestinal wall inflammation and a key additional indicator of the inflammatory process. Preterm infants with a VLBW or ELBW are at a risk of developing more severe FI and NEC. Therefore, the present study aimed to assess the possible role of the gut microbiota and FC levels in the development of inflammatory bowel changes and NEC analysis in underweight preterms with FI.

Subjects and methods

Study design, area and period. The present prospective cohort study was approved by the Local Ethics Commission of Asfendiyarov Kazakh National Medical University (Minutes no. 14 of October 28, 2021). The study included preterm infants with a VLBW and ELBW observed in the Neonatal ICU of the Almaty City Perinatal Center (Almaty, Kazakhstan) from October, 2021 to October, 2022.

Inclusion and exclusion criteria. The inclusion criteria were preterm infants with a birth weight <1,500 g (VLBW or ELBW); an intolerance to enteral feeding (for the study group), and those >14 days after birth. The exclusion criteria were those with congenital intestinal malformations and other severe congenital malformations, and those with incomplete laboratory data, failure to comply with sample collection, storage, and delivery procedures.

Of the 97 preterm infants, 19 were excluded due to poor specimen collection (insufficient stool volume, late delivery and/or violation of storage conditions), which could affect the bacteriological examination results. A total of 78 patients participated in the study: A total of 57 in the study group and 21 in the control group. The average age of the infants was 35 days. Children in the study group presented with signs of GI disorders, including bloating (flatulence), regurgitation, loose stools with mucus and stool discoloration, as well as insufficient weight gain or weight loss. The control group consisted of preterm infants with a birth weight of 1,000-1,500 g with no clinical signs of GI dysfunction (no regurgitation, no symptoms of flatulence, adequate daily weight gain).

All participants in the study received breast milk expressed by the mother, administered via an orogastric tube. All babies received initial antibiotic therapy, as well as caffeine citrate and glucocorticosteroids to prevent and treat bronchopulmonary dysplasia. Ampicillin was administered at a starting dose of 50 mg/kg every 12 h and gentamicin at 5 mg/kg every 48 h for 8-12 days of life. Caffeine citrate was administered as a loading dose of 20 mg/kg followed by a maintenance dose of 5-10 mg/kg/day once daily until clinical resolution of apnea of prematurity. Dexamethasone was administered according to the following tapering regimen: 0.15 mg/kg/day on days 1-3, 0.10 mg/kg/day on days 4-6, 0.05 mg/kg/day on days 7-8, and 0.02 mg/kg/day on days 9-10. None of the patients received probiotics, antacids, or other medications that may affect the GI tract.

During the study, 10 participants (17.5%) in the study group experienced deterioration in clinical condition and developed signs of NEC, including progressive abdominal distension, symptoms of intestinal paresis, and characteristic X-ray changes. These patients were allocated to a separate subgroup with established NEC. The study group design is illustrated in Fig. 1.

Samples and data collection. The study objects were the feces of newborns with a VLBW or ELBW or who were treated in the neonatal ICU and included in the study due to clinical symptoms of FI. Control samples were obtained from premature newborns with a VLBW or ELBW without the clinical symptoms of FI.

The material was collected immediately following the physiological act of defecation, the inner layer of a baby diaper using a sterile spatula, and placed into two sterile containers. Container no. 1 contained Cary-Blair transport medium (Cary-Blair Medium: HiMedia Laboratories) for microbiological investigations (culture, quantitative analysis of microbiota). Container no. 2 did not include a nutrient medium for determining the FC level. The containers had a tightly closing lid and a built-in spatula. Each sample was labeled with an individual patient code, date and the time of collection.

Samples were collected no later than 5 min following defecation to minimize the possible absorption of the liquid stool into the diaper and to avoid the degradation of biomarkers, including calprotectin. To ensure proper processing, the samples were stored in a refrigerator at 2 to 4˚C for no more than 2-3 h prior to transport to the laboratory. The samples were delivered to the laboratory in a special cooling bag, ensuring a stable temperature.

In the laboratory, samples from container no. 1 were incubated in a thermostat at +37˚C for 4-7 days. Colony growth was then assessed, and the microbial load was determined (in CFU/g). Samples from container no. 2 were analyzed using ELISA for FC levels in the stool extract (in μg per 1 g of feces).

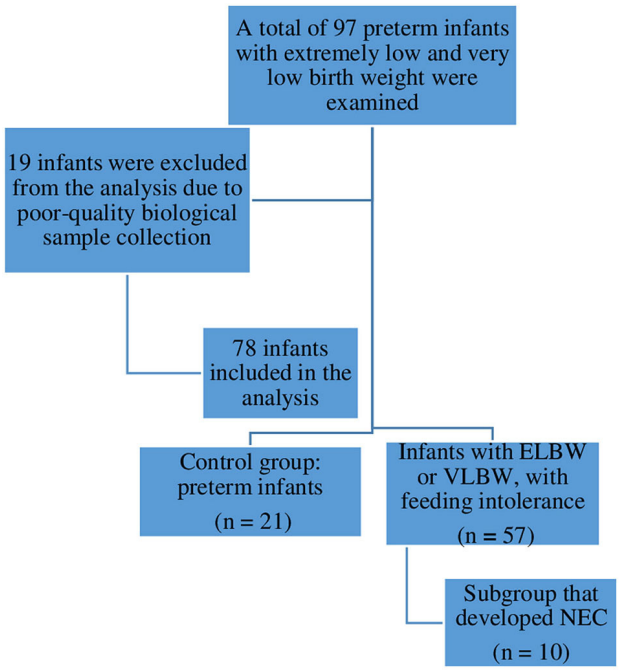

Figure 1. Study and group design.

Such an approach to collecting and transporting samples allowed for the maintenance of the integrity of the microbial composition and ensuring the reliability of determining FC levels, which is critical when working with newborns and small volumes of biomaterial.

The bacteriological culture of feces and determination of fecal calprotectin (FC) levels were performed on a contractual basis in the accredited commercial laboratory 'Invivo.kz' (Almaty, Kazakhstan; Contract no. 230879 dated October 5, 2022). All analyses were carried out using standard validated methods in accordance with the manufacturers' instructions. Stool samples were cultured on Salmonella-Shigella agar (SS agar; HiMedia Laboratories Pvt. Ltd.) and analyzed using the MicroScan WalkAway 96 Plus automated microbiology system (Beckman Coulter, Inc.) for bacterial identification.

Fecal calprotectin concentrations were determined using a Calprotectin ELISA kit (cat. no. EQ 6831-9601 W, EUROIMMUN Medizinische Labordiagnostika AG) on an ImmunoChem-2100 microplate photometer (High Technology, Inc.), with all analyses performed according to the manufacturers' validated protocols.

Statistical analysis. Statistical analysis was performed using STATISTICA, version 12 (StatSoft Inc., USA). Baseline clinical and demographic characteristics were compared between two independent groups (healthy preterm infants and preterm infants with feeding intolerance). Continuous variables (gestational age and birth weight) were expressed as mean ± standard deviation (SD) and compared using the independent samples Student's t-test. Categorical variables (sex, mode of delivery, use of antenatal steroids, etc.) were compared using Pearson's χ^2 test. The composition of the intestinal microbiota was compared in the same two independent groups. Fisher's exact test was used to compare the proportions due to the small number of some cells included in the contingency analysis (<5). Fisher's exact test was also used to assess the association between the clinical group and FC levels. Statistical significance was assessed using the Monte Carlo method (10,000 simulations), as the expected number of observations was <5 in >20% of cells. Pearson's χ^2 test was also used and data are presented as a descriptive measure of the association. All tests were two-tailed and a P-value <0.05 was considered to indicate a statistically significant difference. Correspondence analysis was conducted to visualize the association between clinical groups and FC categories. This method is based on the χ^2 statistic and represents the rows and columns of the contingency table as points in a low-dimensional space.

Results

In the present study, 78 preterm infants with a birth weight <1,500 g were included, comprising 21 healthy preterm infants and 57 preterm infants with feeding intolerance. The overall clinical and demographic characteristics are summarized in Table I. There were no statistically significant differences between the groups with respect to sex, mode of delivery (cesarean section or vaginal delivery), or use of antenatal steroids (P>0.05 for all comparisons). However, infants with feeding intolerance had a significantly lower gestational age and birth weight compared with healthy preterm infants (26.0±1.9 vs. 28.0±1.03 weeks and 798±137 g vs. 1,190±102 g, respectively; both P<0.001).

Microbiological tests revealed fairly diverse microbial associations in the intestines of the examined premature newborns (Fig. 2). The initial microbiological landscape represents pooled data rather than comparative analysis across subgroups.

The microbial landscape was dominated by normal flora at a concentration of at least 10^7-10^8 CFU/g, including *Bifidobacterium spp*. in 78 (100%) of the infants and *Lactobacillus spp*. in 68 (87.2%) of the infants; *Escherichia coli* with normal enzymatic activity was observed in 44 (56.4%) of the infants and with reduced enzymatic activity in 27 (34.6%) of the infants. The Gram-positive lactic acid microorganism, *Enterococcus faecalis*, a component of the normal microflora of the gastrointestinal tract in breastfed newborns, was found in 36 (46.2%) of the infants, and *Staphylococcus epidermidis* in 17 (21,8%) of the examined infants. The remaining opportunistic flora was observed in isolated cases (Fig. 2).

Notably, 57/78 (73%) of the preterm infants presented pathogenic intestinal flora that can impair the GI tract function and promote the development of FI symptoms. The normal flora composition was almost similar in the study group and the control group; however, only children with FI had pathogenic flora within 10^7-10^8 CFU/g (Fig. 3).

Statistical analysis revealed a statistically significant difference in the presence of *Escherichia coli* with normal enzymatic activity, *lactobacilli* and pathogenic flora between the healthy preterm infants and preterm infants with FI. The comparative analysis using Fisher's exact test revealed statistically significant differences in the intestinal microbiota composition between the study groups (Table II). In the preterm infants with FI, lactobacilli were less frequent compared with the healthy preterm infants (82.4 vs. 100%; P=0.039 analyzed using Fisher's exact test), as were *Escherichia coli* with

Table I. Baseline characteristics of the infants included in the study.

Characteristic	Healthy preterm infants, n=21	Preterm infants with feeding intolerance, n=57	P-value
Male sex, n (%)	10 (47.6%)	29 (50.9%)	0.81
Female, n (%)	11 (52.4)	28 (49,.1%)	0.81
Cesarean delivery, n (%)	9 (42.9%)	27 (47,.4%)	0.71
Antenatal steroids, n (%)	13 (61.9%)	30 (56.1%)	0.64
Birth weight, g, mean ± SD	1,190±102	798±137	<0.001
Gestational age, weeks, mean ± SD	28.0±1.03	26.0±1.9	<0.001

Data are presented as n (%) or mean ± SD. P-values were obtained using Pearson's χ^2 test or the independent samples Student's t-test (two tailed); P<0.05 was considered statistically significant.

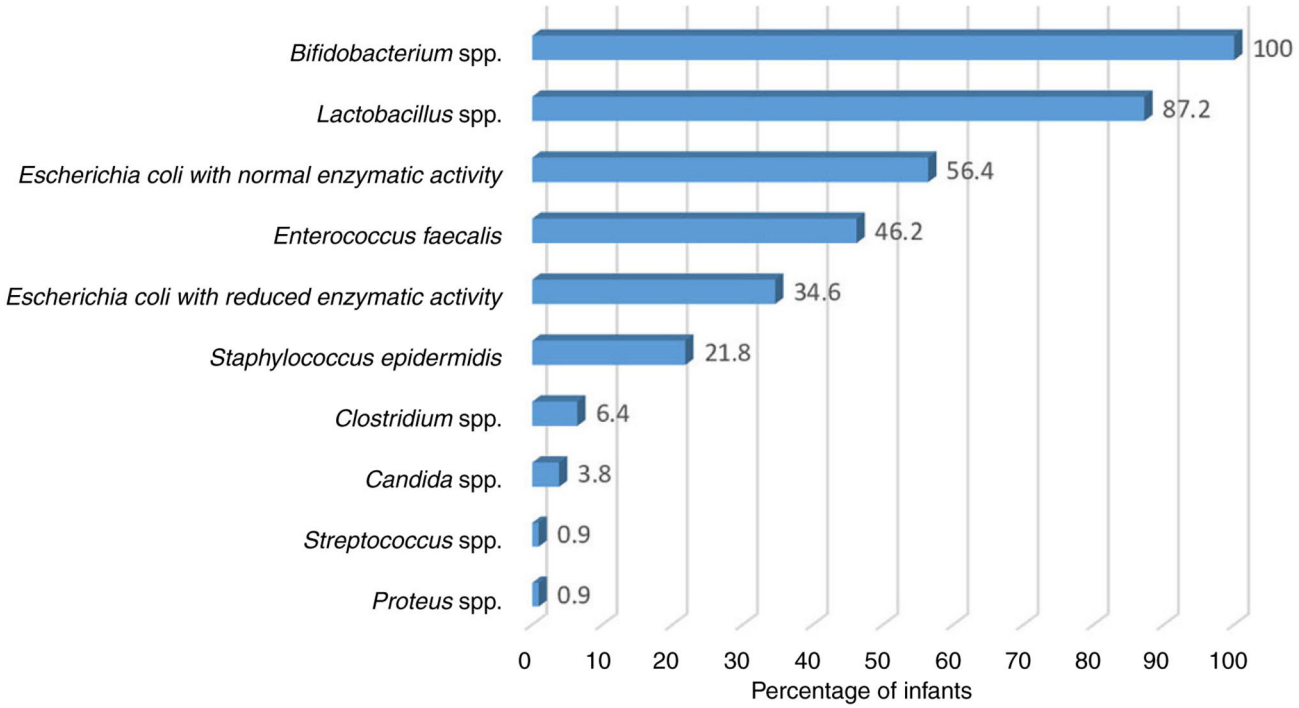

Figure 2. Intestine microbiological profile in preterm infants with a very low birth weight and an extremely low birth weight (n=78). Data are presented as percentages (%).

normal enzymatic activity (23.8 vs. 68.4%; P=0.001). At the same time, preterm infants with FI had a higher proportion of opportunistic microorganisms. Pathogenic flora were present in all infants with FI (100%) and absent in the healthy preterm infants (P<0.001 analyzed using Fisher's exact test), indicating a markedly more pronounced dysbiosis in the study group.

The analysis confirmed a markedly higher colonization of the GI tract by pathogenic microflora in the newborns with FI. In the present study, the pathogenic microflora in the newborns with FI (n=57) was presented by *Klebsiella pneumoniae* in 17 (29.8%) of the infants at 10^7 CFU/g, *Klebsiella oxytoca* in 13 (22.8%, 10^7 CFU/g), *Enterobacter cloacae* in 10 (17.6%, 10^7 CFU/g), *Enterobacter aerogenes* in 9 (15.8%, 10^8 CFU/g), *Citrobacter koseri* in 6 (10.5%, 10^7 CFU/g), *Candida albicans* in 4 (7.0%, 10^5 CFU/g) and *Acinetobacter baumannii complex* in 1 (1.7%, 10^8 CFU/g) of the infants (Fig. 4). Such a pronounced colonization of the intestine with pathogenic and opportunistic flora may have clinical significance for the development of food intolerance in this patient category and may indicate a disturbance in the microbiocenosis.

The measurement of FC levels using ELISA in stool samples was used as a non-invasive marker to quantify the intestinal condition in infants with a VLBW or ELBW. This method was safe and simple, and allowed the quantitative determination of FC levels expressed in μg/g. As illustrated in Fig. 5, FC levels in preterm infants were consistent with their condition. The majority of healthy preterm infants (n=21; 47.6 and 52.4%, respectively) had low to moderate FC levels (50-350 μg/g), with none >350 μg/g. In preterm infants with FI (n=47), low and moderate levels prevailed (59.6 and 21.3%, respectively); however, some had elevated calprotectin concentrations (≥350 μg/g: 8.5% with 350-500 μg/g and 10.6% with

Table II. Comparison of the intestinal microbiota in healthy preterm infants and preterm infants with feeding intolerance.

Microorganisms	Healthy preterm infants, % (n=21)	Preterm infants with feeding intolerance, % (n=57)	P-value
Bifidobacteria	100.0	100.0	0,.999
Lactobacilli	100.0	82.4	0.039[a]
Escherichia coli with normal enzymatic activity	23.8	68.4	0.001[b]
Escherichia coli with reduced enzymatic activity	23.8	38.8	0.217
Enterococci	57.1	42.1	0.238
Staphylococcus epidermidis (S. epidermidis)	28.5	19.3	0.383
Pathogenic microorganisms	0.0	100.0	<0.001[b]

Data are presented as percentages (%).P-values were obtained using Fisher's exact test (two-tailed); [a]P<0.05; [b]P<0.01.

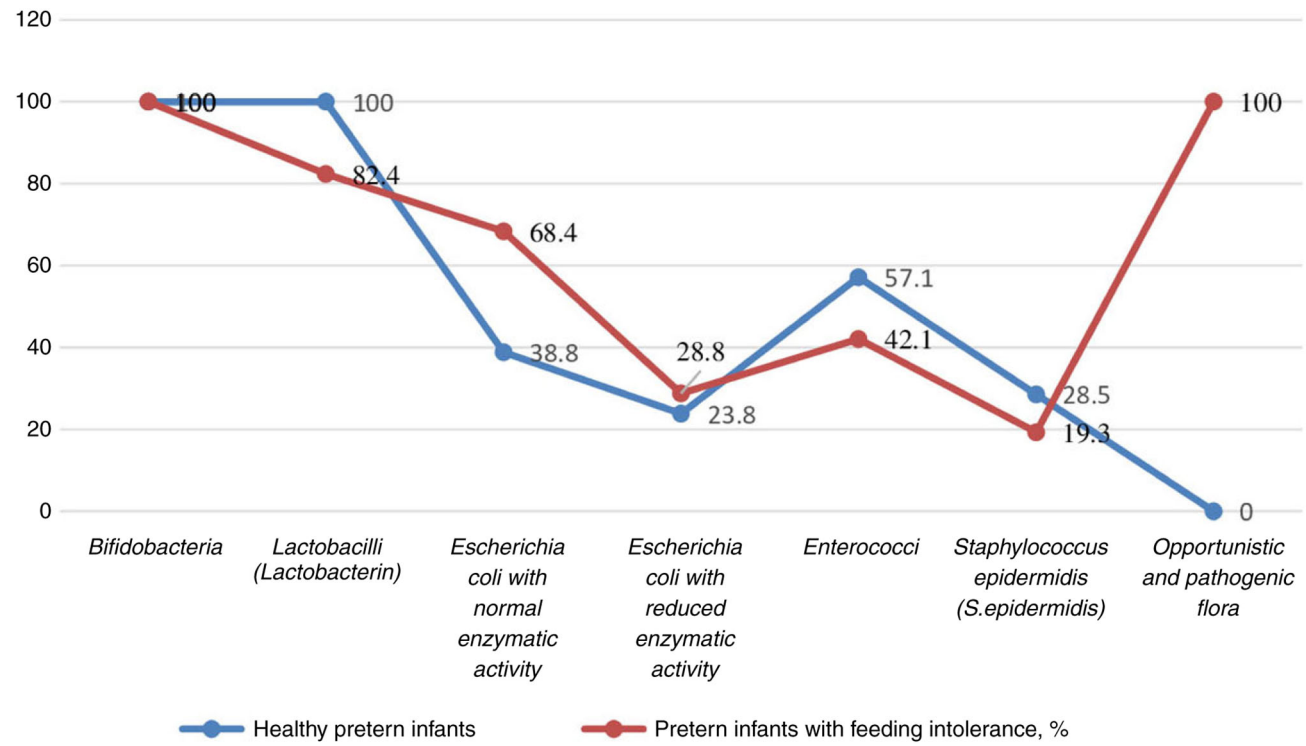

Figure 3. Intestinal microbiota composition in healthy preterm and preterm infants with feeding intolerance (n=78). Data are presented as percentages (%).

>500 μg/g). The most pronounced increase was observed in the preterm infants with NEC (n=10): All had high FC levels (≥350 μg/g), with 40.0% in the 350-500 μg/g range and 60.0% exceeding 500 μg/g, reflecting a significant increase in intestinal inflammation in NEC. Thus, in the study groups, FC levels, evaluated against the background of microbiological changes, were associated with identified disturbances of the gut microbiota (dysbiosis).

The association between the clinical group and FC levels was evaluated using Fisher's exact test for an R x C contingency table with the Monte-Carlo estimation (10,000 simulations), which demonstrated a statistically significant association (P<0.001). The NEC group had the highest proportion (60.0%) of infants with markedly elevated FC levels (>500 μg/g), compared with 10.6% among preterm infants with feeding intolerance. No FC elevation was observed among the healthy preterm infants, in whom FC levels were not >350 μg/g (Table III).

The association between clinical groups and FC categories is presented in Fig. 6, as determined by correspondence analysis based on χ^2-derived distances. For this analysis, the same contingency table as in Table III was used: χ^2 distances between row and column profiles of this table were calculated and then plotted in a reduced two-dimensional space. A sharp angle indicates a positive association with FC levels >500 μg/g. The plot illustrates a statistically significant association [$\chi^2(6)=43.536$; P<0.001]. The obtained results demonstrate that unfavorable intestinal colonization patterns in preterm infants (a dysbiotic shift toward pathogenic flora) are associated with elevated FC levels. This confirms the potential diagnostic and differential diagnostic value of FC levels for identifying

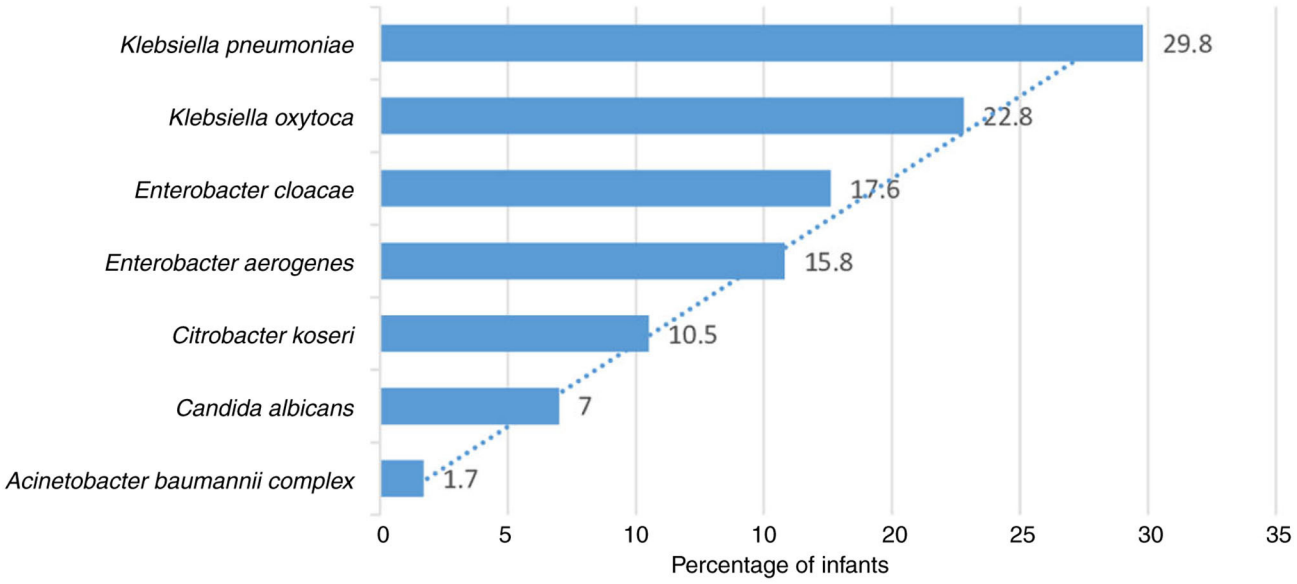

Figure 4. Pathological intestinal microbiota in ELBW and VLBW infants with feeding intolerance (n=57). Data are presented as percentages (%).

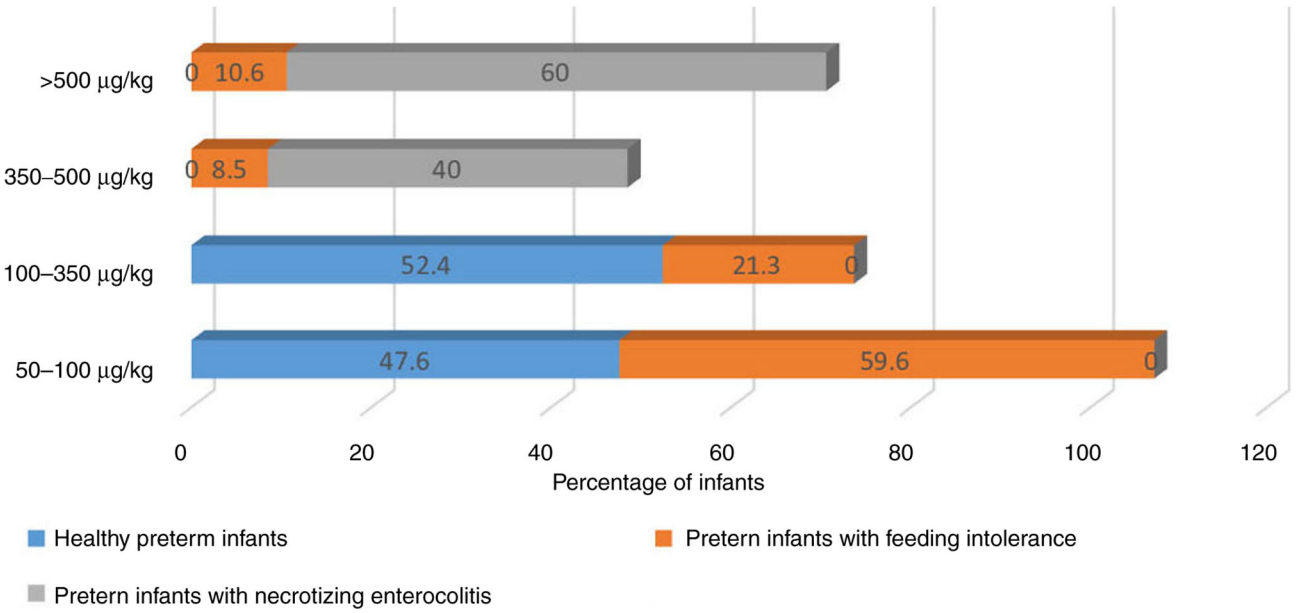

Figure 5. Fecal calprotectin levels in preterm infants with a very low birth weight and an extremely low birth weight. Data are presented as percentages (%).

and stratifying the severity of inflammatory bowel disease in preterm infants, particularly for differentiating between functional FI and NEC.

Discussion

FI and NEC are fundamentally different, yet clinically similar conditions in preterm infants. FI often reflects the functional immaturity of the GI tract and dysbiotic changes, whereas NEC is a severe, inflammatory, and necrotic intestinal condition with a high risk of adverse outcomes. Since both FI and NEC are closely dependent on the specifics of intestinal microbial colonization, qualitative and quantitative studies of the microbiota composition in preterm infants are particularly important. The identification of bacterial (and fungal) infections allows for the characterization of the intestinal biocenosis and the identification of potential pathogens associated with NEC development.

In the present study, a comparative analysis of the intestinal microbiological profile in the examined newborns revealed that representatives of the *Enterobacteriaceae* family, including *Klebsiella pneumoniae* and *Enterobacter cloacae*, as well as fungal flora (*Candida albicans*), predominated in the fecal cultures of children with NEC. These results align with those of prior publications on the microbiota in preterm infants. For example, Coleman *et al* (38) reported a predominance of *Klebsiella* spp. in the intestines of infants who developed NEC compared with a predominance of commensal *Escherichia* spp. in healthy neonates. In addition, a metagenomic study in India also revealed a significant increase in the proportion

Table III. Distribution of fecal calprotectin levels in preterm infants by clinical group.

Clinical group	50-100 μg/kg	100-350 μg/kg	350-500 μg/kg	>500 μg/kg	Total
Healthy preterm infants	10 (47.6%)	11 (52.4%)	0 (0%)	0 (0%)	21
Preterm infants with feeding intolerance	28 (59.6%)	10 (21.3%)	4 (8.5%)	5 (10.6%)	47
Preterm infants with necrotizing enterocolitis	0 (0%)	0 (0%)	4 (40.0%)	6 (60.0%)	10
P-value	0.002	0.003	0.006	0.0001	

Data are presented as n (%). P-values were obtained using two-tailed Fisher's exact test; P<0.05 was considered statistically significant.

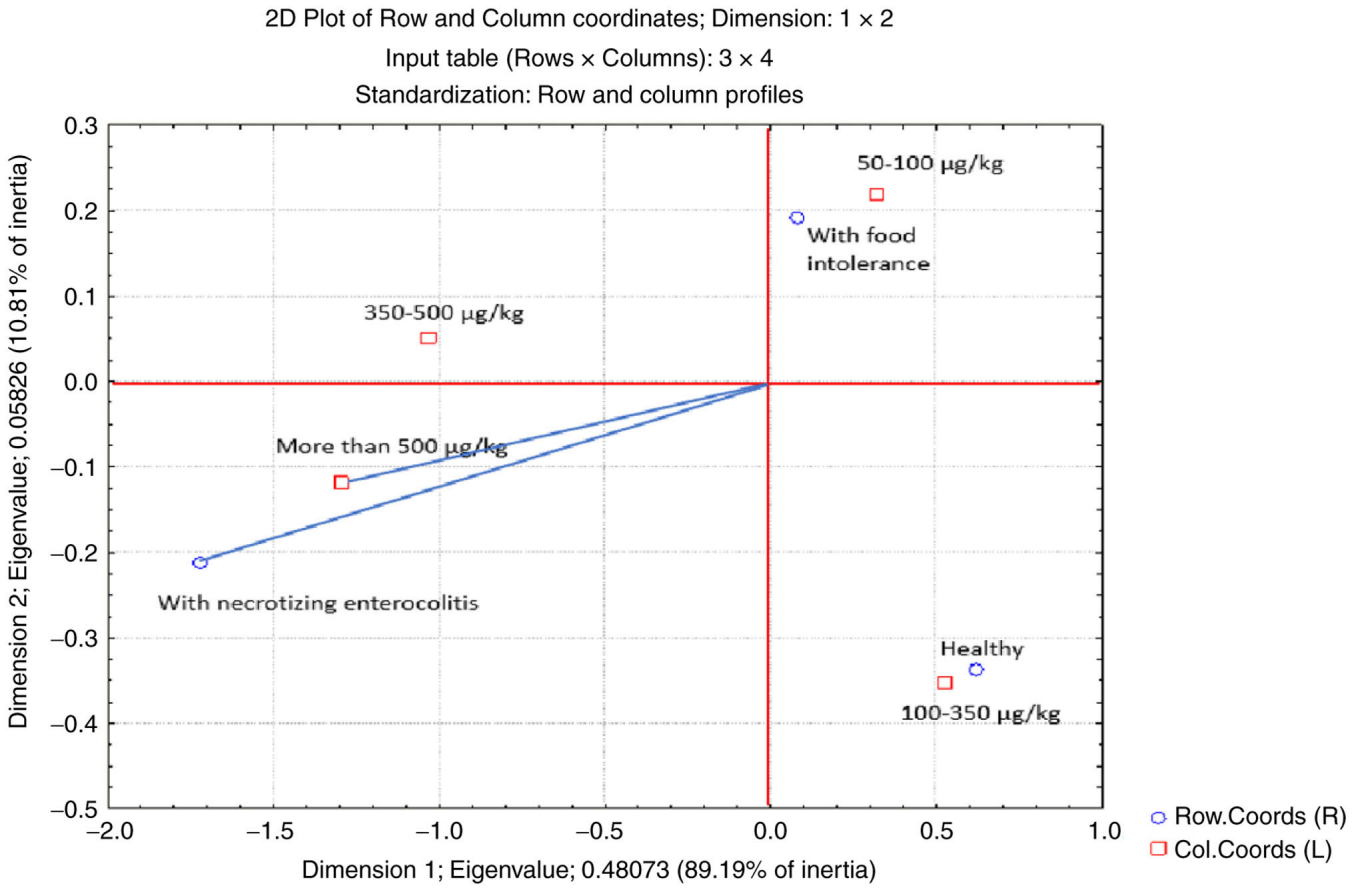

Figure 6. Correspondence analysis plot. Associations between preterm infant groups and calprotectin ranges.

of *Enterobacteriaceae* and the presence of virulence genes associated with impaired intestinal barrier function (39).

Harmful bacteria, including *Enterococcus*, *Klebsiella* and *Acinetobacter,* dominate the intestinal microflora of premature infants. Premature infants with an ELBW have less beneficial bacteria and more potentially harmful bacteria in the gut. These potentially harmful bacteria may serve as potential biomarkers of intestinal dysbiosis and increased risk of NEC in preterm infants with an ELBW.

The role of *Candida* in the development of NEC in preterm infants remains poorly understood, despite several studies confirming its involvement in the condition. Thus, according to the study by Pappas *et al* (40), 25% of premature babies with prolonged antibiotic use presented with *Candida*, as well as *Klebsiella* and *Enterococcus*. Dermitzaki *et al* (41) revealed that newborns, particularly preterm infants, admitted to ICUs, had pronounced risk factors for invasive *Candida* infections, and the use of antifungal prophylaxis is particularly justified for this category of patients. It has been reported that *Acinetobacter baumannii* has become a common pathogen causing hospital-acquired infections worldwide (24,42). As a typical nosocomial pathogen, it can colonize the skin and GI tract of humans (42). Currently, *Acinetobacter* spp. increasingly causes severe illness in newborns worldwide. The reason for such an escalation is unclear; however, it may be related to a combination of host, pathogen and environmental factors (43).

In previous a review of factors influencing the gut microbiota in newborns, Srinivasjois *et al* (44) identified several key determinants of microbiota formation: The effects of antibiotics, feeding type, probiotic use and prolonged infant stays in neonatal ICUs. Preterm infants are particularly susceptible to intestinal dysbiosis, which is associated with conditions such as NEC, growth retardation, cognitive impairments and brain damage (45). Accumulated data indicate that functional FI and NEC in preterm infants are associated with severe disturbances in intestinal microbial colonization and activation of the local inflammatory response.

This places special emphasis on non-invasive diagnostic methods, allowing for the timely detection of early signs of inflammation without traumatic interventions. FC reflects the local inflammatory response of the intestinal mucosa. Determining the FC level is a safe and convenient method for early diagnosis of inflammatory and necrotic intestinal lesions, especially in preterm infants, in whom invasive procedures are limited by clinical condition. The present study used FC as a non-invasive marker confirming inflammatory changes in the intestines of the examined newborns. FC levels were reliably higher in infants with suspected NEC than in infants with signs of functional GI disorders and in the control group.

Recent foreign studies have reported similar data. A previous study demonstrated that FC levels were statistically significantly higher in preterm infants with NEC than in the control group; they were directly associated with the severity of inflammatory changes in the intestinal mucosa (46).

In another study, an elevated FC level was used as an early diagnostic criterion for NEC. In the NEC group, FC levels were higher than in the FI group and healthy children ($P<0.05$); FC ranged from 250 to 400 μg/g in children with mild inflammation and exceeded 800 μg/g in children with severe NEC (47), which is comparable with the results of the present study.

FC levels may also depend on microbiota composition. In a previous study, children with a lower gestational age (<32 weeks) exhibited a greater variability in FC and inflammatory metabolite levels (48). This could be due to the immaturity of the gut microbiota (48). These results are consistent with the observations of the present study: Extremely preterm babies (>32 weeks) had higher FC levels than healthy preterm infants, suggesting varying degrees of intestinal colonization and immune response development.

The strengths of the present study included a well-defined cohort of extremely preterm infants with a VLBW of ELBW, a unified feeding strategy based exclusively on expressed breast milk, and a combined assessment of gut microbiota using culture methods and FC levels as an objective, non-invasive marker of intestinal inflammation. However, the present study had some limitations which should be mentioned. First, the single-center, observational nature of the study and a relatively small sample size (78 premature infants, including 10 who developed NEC) limit the statistical power of the subgroup analysis and the generalizability of the results. Second, although the groups were comparable by sex and method of delivery, the authors did not systematically collect or adjust for important maternal and perinatal factors (e.g., maternal infections, prenatal antibiotic exposure, chorioamnionitis, overall disease severity). Third, all infants received empirical antibiotic therapy, caffeine citrate and glucocorticoids, and the isolates were not tested for antibiotic susceptibility. Finally, the present study did not provide a detailed description or systematic analysis of the complete clinical picture (including the evolution of gastrointestinal and systemic symptoms and the severity of the disease); the main focus was on microbiological data and FC levels.

In conclusion, the present prospective observational study of the gut microbiota revealed the presence of pathogenic flora represented by *Klebsiella pneumoniae* (29.8%), *Klebsiella oxytoca* (22.8%), *Enterobacter cloacae* (17.6%), *Enterobacter aerogenes* (15.8%), *Citrobacter koseri* (10.5%), *Acinetobacter baumannii complex (*1.7%) and *Candida albicans* fungi (7.0%) in preterm infants with a VLBW or ELBW and with FI (n=57) compared to healthy preterm infants. A total of 10 of the 57 infants (17.5%) subsequently developed NEC, and a pronounced inflammatory process in the intestine was accompanied by an increase in FC level. Disturbances in the intestinal microbiocenosis should be considered a key pathogenetic risk factor for the development of NEC in preterm infants with a VLBW or ELBW and with FI in ICUs. For preterm infants with a VLBW or ELBW in ICUs, the early identification of pathogenic gut colonization and elevated fecal calprotectin may help stratify the risk of FI and NEC and guide targeted preventive strategies.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

GB conceived and designed the study, collected data, drafted the initial version of the manuscript, and critically reviewed and revised the manuscript. KZ collected data and critically reviewed and revised the manuscript. GX assisted with the statistical analysis and critically reviewed the manuscript. AK provided consultations on the interpretation of the laboratory tests and contributed to the literature search related to the study. MA and NV collected and transported biological samples, entered patient data into Excel spreadsheets, and managed the databases. KZ and GX confirm the authenticity of all the raw data. All authors have read and approved the

final version of the manuscript and agree to be accountable for all aspects of the work.

Ethics approval and consent to participate

sThe present study was conducted from August, 2022 to December, 2023 and was approved by the Local Ethics Commission of the Kazakh National Medical University. S.D. Asfendiyarov (Ethical no. 1331 dated October 28, 2021).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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