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INTESTINAL MICROFLORA IN CHILDREN WITH INFANTILE COLIC

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Functional gastrointestinal disorders are the most prevalent pathological conditions in children. Infantile colic is the most common problem during the first months of life. One of the possible mechanisms that could be pathogenically involved in the occurrence of the infantile colic is changes in intestinal microbiota. This research aims to study the gut microbiota in children with infantile colic. We examined gut microflora of 57 children (aged from 1 to 5 month) with intestinal colic. It was found that children with infantile colic have gut microbiome disorder that is manifested in decreased numbers of obligate anaerobes, increased growth of facultative anaerobes of the Proteobacteria phylum, mainly species of the Enterobacteriaceae family, increased number of microorganisms of the Firmicutes phylum, especially of the Staphylococcaceae family, and increased numbers of Candida species. An increased number of gram-negative (*K. pneumoniae*) and gram-positive (*S. aureus*) opportunistic microorganisms characterized gut microbiome in children with infantile colic. *Candida* species and *S.aureus* were the most common pathogens to colonize the gut. Among the frequently seen microbial communities, three- and two-component ones prevailed. Most commonly *Candida* species formed communities with *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*. Indirect evidence of gut redox potential shift to the acid side in children with infantile colic are the emergence of opportunistic flora that is usually cultivated in neutral or acidic environments (*Candida* species, EHEC) and reduction of the numbers of microflora representatives that produces organic acids (commensal *E.coli*, Lacto- and Bifidumflora).

Key words: infantile colic, intestinal microbiocenosis, opportunistic microorganisms, *Candida*, gut microbiome, gut redox potential.

С.С. Левенець, Н.М. Горобець, Г.Д. Кіржнер, В.Є. Сабадаш МІКРОФЛОРА КИШКІВНИКА У ДІТЕЙ З ДИТЯЧОЮ КОЛЬКОЮ

Одним з найпоширеніших станів серед дітей перших місяців життя є функціональний шлунково-кишковий розлад - дитяча колика. Серед багатьох можливих механізмів, які можуть бути патогенетично причетні до виникнення дитячої коліки, на даний час розглядаються зміни в мікрофлорі кишківника. Тому метою дослідження було вивчення мікробного пейзажу кишківника у дітей з дитячою коликою. Обстежено мікрофлору товстої кишки у 57 пацієнтів з дитячою коликою віком від 1 до 5 місяців. Встановлено, що у дітей з коликою спостерігається порушення в мікробіомі кишківника, що виявляється у зменшенні кількості облігатних анаеробів та збільшенні росту факультативних анаеробів типу Proteobacteria, переважно членів родини Enterobacteriaceae, збільшенні кількості мікроорганізмів типу Firmicutes, особливо родини Staphylococcaceae та збільшення кількості грибів роду *Candida*. Також при коліці в мікробіомі збільшується кількість умовно - патогенних мікроорганізмів грамнегативних (*K. pneumoniae*) та грампозитивних (*S. aureus*). Найпоширенішими збудниками, що колонізували кишківник при інфантильній коліці були Гриби роду *Candida* (*C.albicans*) і золотистий стафілокок (*S.aureus*). Серед спільнот, які контамінували кишківник, переважали три - та двокомпонентні асоціації умовно-патогенних мікроорганізмів та грибів. Найчастіші асоціації грибів роду *Candida* відмічалися з *S.aureus*, *K.pneumoniae* та *E.faecalis*. Опосередкованим свідченням зміщення редокс-потенціалу кишківника в кислу сторону у дітей з коликою є поява в мікробіомі кишківника умовно-патогенної флори, культивування якої відбувається в нейтральних або кислих середовищах (гриби роду *Candida*, ентерогеморагічна кишкова паличка) та зменшення кількості представників мікрофлори, яка виробляє органічні кислоти (комменсальна *E.coli*, Лакто- та Біфідумфлора).

Ключові слова: дитяча колика, мікробіом кишківника, умовно-патогенні мікроорганізми, гриби роду *Candida*, редокс-потенціал кишківника.

The work is initiative.

Functional gastrointestinal disorders (FGID) are the most common problem in children in the first months of life. In this condition, there are any organic changes in the gastrointestinal tract (structural abnormalities, inflammatory changes, infections or tumours) and metabolic abnormalities [14]. FGID may change intestinal motility, digestion and absorption of nutrients, as well as the intestinal microbiota composition and the immune system activity. The reasons of functional disorders often lie outside the affected organ and are caused by a violation of nervous and humoral regulation of the digestive tract.

Baby colic leads to excessive crying and is often the cause of visits to the paediatrician at the age of 2 weeks to 3 months [8]. It is characterized by bouts of irritability, crying, accompanied by clenched fists, legs clenched, redness. It usually begins in the second or third week after birth with a peak of clinical manifestations at the age of 5–8 weeks. It usually stops spontaneously at the age of 4–5 months. At present, Roman criteria IV [9] are used to diagnose neonatal colic, including:

- paroxysms of irritability with anxiety or crying, which begin and stop for no apparent reason;
- symptoms of colic lasting 3 or more hours a day and lasting 3 or more days a week longer than 1 week;
- thriving and disappearance of colic symptoms in infants from birth to 5 months of age.

The pathogenesis of infantile colic is still not fully understood. It is associated with three hypothetical theories: immaturity of the mechanisms of action of bile acids, intestinal peristalsis and changes in the microbiota. Another reasons are called changes in the intestinal microflora and hormones, gas formation, allergy to cow's milk products, increase in the age of motherhood, first pregnancy, smoking of mothers, problems of parental behavior (family disagreements, anxiety of parents) [8].

One of the hypothetical mechanisms that is pathogenetically involved in the occurrence of baby colic is changes in the gut microbiome [9]. Its microflora is formed as a result of the processes occurring both in a large intestine, and in all gastrointestinal tract and therefore reflects the general condition of a macroorganism [6].

The purpose of the study was to access the changes in the microbiome of the large intestine in children with intestinal colic.

Materials and methods. In our research, we studied colon microflora of 57 children with colic. Children's age was from 1 month to 5 months, and gender balance was 39 (68 %) boys and 18 (32 %) girls, respectively. Patients' parents gave written informed consent to share information. This study was performed according to the Council of Europe Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine (ETS No. 164) of 04.04.1997, and the World Medical Association Declaration of Helsinki (2008).

Biological material for research has been collected and transported according to the requirements, in the environments, registered and certified in Ukraine.

Analysis of faecal microbiota dysbiosis was done according to the examination method developed by R.V. Epstein-Litvak (1977) and is based on the quantitative calculation of bacteria found in the dilution of 1 g of faeces that were inoculated on the selective nutrient environment. The rate of microbial colonization intensity (microbial number) was determined by counting colonies (colony forming units (CFU)). Bergie's classification was used to identify various microorganisms [11].

The statistical processing of the data was carried out using the electronic package Microsoft Excel 7.0 and R studio. In this case, correlations between different microbial species that contaminated the gut were determined. The nonparametric Mann-Whitney test was used to determine the statistical significance of differences between the groups. The difference was considered statistically significant at $p < 0.05$.

Results of the study and their discussion. Among children with intestinal colic, opportunistic flora was found in 56 (98 %) children, being absent in one child (2 %). With greater frequency, microorganisms were sown in associations. Three- (25–46 %) and two-component (18–32 %) associations prevailed. In a small number of children, feces was contaminated with only one opportunistic pathogen (7–12 %). Five (2–3 %) and four-component (3–5 %) associations were observed even less often.

Candida was found in 34 (59 %), microorganisms of phylum Firmicutes in 45 (79 %) species and phylum Proteobacteria in 29 (51 %) (fig. 1).

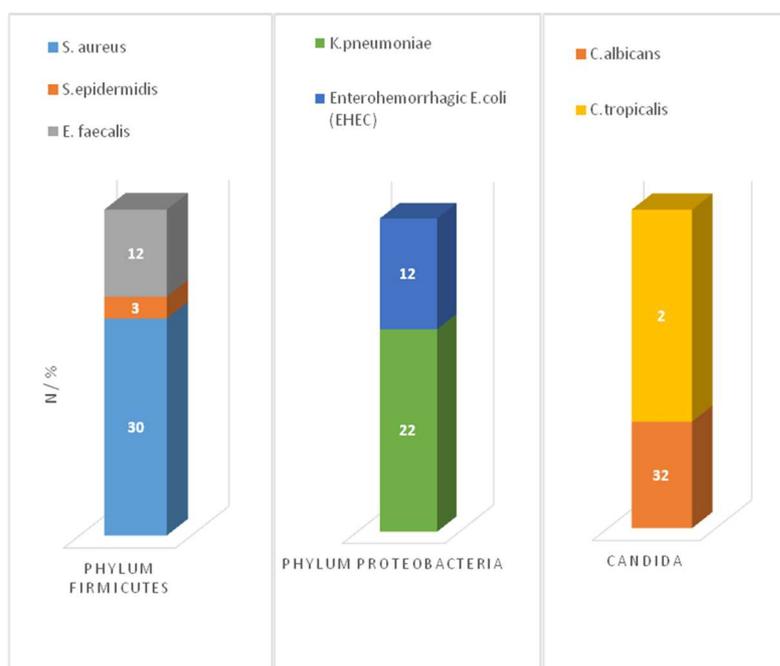


Fig. 1. Intestinal microbiome composition in children with infantile colic.

Among the pathogens of the family Staphylococcaceae was dominated by *S. aureus*, which was sown in half of children 30 (53 %). *S. epidermidis* occurred with a low frequency – 3 (5 %). A member of the family Enterobacteriaceae, *K. pneumoniae* was found in 22 (39 %) in concentrations of 10^4 to 10^8 CFU/g. Enterohemorrhagic *E. coli* (EHEC) (7–12 %) was sown with low frequency. A decrease in the amount of *E. coli* with normal enzymatic activity was observed in 25 (44 %) of the examined children. The most common pathogens that colonized the intestine in children with colic were the fungi *C. albicans* 32 (56 %) and *Staphylococcus aureus*, *S. aureus* 30 (53 %).

In children with colic for fungi of the genus *Candida* is characterized by associative growth (Table 1).

Table 1

Candida in microbial communities in children with colic

Microbial species	Number of species in microbial communities/frequency				
	2	3	4	5	Total n/%
<i>E. coli</i> (atypical)	2	12	2	1	17 (30 %)
<i>S. aureus</i>	5	6	4	1	16 (28 %)
<i>E. faecalis</i>	2	11	2	1	16 (28 %)
<i>K. pneumoniae</i>	2	7	4	1	14 (25 %)
<i>Citrobacter freundii</i>		1			1 (2 %)
<i>P. aeruginosa</i>		1			1 (2 %)

They were sown only in associations with other opportunistic pathogens, not found in the mono form. They occurred with the highest frequency in three – 6 (10 %) and two component – 4 (7 %) associations. With a small frequency (2 %; 3 %), they were included in the five and four component associations, respectively.

Fungi of the genus *Candida* were found almost equally often in combination with *Staphylococcus aureus* – in 16 (28 %), *K. pneumoniae* – 14 (25 %), less often – with *E. faecalis* – 8 (14 %) children. In all cases of seeding enterohemorrhagic *E. coli* – 6 (10 %), it was combined with fungi of the genus *Candida*. Fungi with *P. aeruginosa* 1 (2 %) and *Citrobacter freundii* 1 (2 %) occurred with a low frequency.

The most common combinations of microorganisms:

Candida + *E. coli* (EHEC) + *E. faecalis* – 6 (10 %);

Candida + *S. aureus* – 5 (9 %);

Candida + *K. pneumoniae* + *E. faecalis* – 5 (9 %);

Candida + *S. aureus* + *E. coli* (EHEC) – 4 (7 %).

The content of obligate microflora, in particular lactobacilli, was reduced in 39 (68 %) children. Of these, patients whose feces were contaminated with fungi of the genus *Candida* were 28 (49 %) (table 2).

Table 2

Number of gut obligate microbiota in communities with *Candida* in children with infantile colic

Obligate anaerobic microflora and <i>Candida</i> as microbial communities	Lactobacillus (CFU/g)		Bifidobacterium (CFU/g)	
	<10 ⁸	≥ 10 ⁸	<10 ⁸	10 ⁸ –10 ¹⁰
Quantity (n/ %)	28 (49 %)	4 (7 %)	22 (39 %)	6 (10 %)

In a small number of children 4 (7 %), whose feces are contaminated with *Candida*, the level of lactobacilli was appropriate. The content of *Bifidobacterium* was reduced in 37 (65 %), of which 22 (39 %) in the case of association with fungi of the genus *Candida*.

Due to the lactobacilli synthesis of organic acids, intestinal pH decreases. Certain low levels of intestinal redox potential inhibit the growth and activity of acid-sensitive groups of microorganisms.

It is known from current scientific data that exometabolites of *C. albicans* slow down the catalase activity of *E. coli* and *S. aureus* strains, which leads to increase in the damaging effect of reactive oxygen species on microorganisms, which may affect the survival and number of microbial associations [2].

Literature data [4] indicate that representatives of bifidum- and lactobacilli shift the pH of the medium to the acidic side (lactobacilli mainly synthesize lactic acid, and bifidumbacteria – lactic and acetic), thereby creating favorable conditions for the growth of fungi of the genus *Candida* (provided they are infected), which in turn enriches the habitat of this group of bacteria with amino acids and vitamins and also synthesize organic acids, which leads to low redox potential [3]. As it is known that the medium acidity for the growth of strains of the genus *Lactobacilli* is in the range of 5.5 to 7.0. The growth of bifidobacteria occurs in a wider pH range: from 4.5 to 8.5 [13]. As a result, it is possible to stop the activity of lactobacilli due to a significant decrease in the intestinal pH during the activity of the fungus *Candida*. While the state of the intestinal redox potential begins to block the activity of lactobacilli and their concentration decreases, bifidumbacteria can still live and multiply. However, their number also decreases, approaching the number of *Lactobacilli* and not making a significant difference between them ($p > 0.05$).

Given this fact, it can be assumed that the decrease in the content of obligate anaerobes in the case of intestinal contamination in children with fungi of the genus *Candida*, is forced to be compensatory in order to protect the intestine from a sharp change in the redox potential. Reducing the number of obligate microflora in the intestines of children depresses their antagonistic potential for virulence and persistence factors of bacterial flora, in particular *Enterobacteria* [5].

Among the representatives of sown *Enterobacteria*, a decrease in the level of commensal *E. coli* in children with intestinal colic was observed in almost half of children – 25 (44 %). Persistence of *Escherichia coli* with reduced enzymatic activity was observed in 10–17 %, enterohemorrhagic (EHEC) in

7–12 %. Commensal *E. coli* is a major competitor to EHEC [12]. Between them there is competition for organic acids, amino acids and other nutrients, the exchange of mobile genetic elements [7].

A common feature of the family Enterobacteriaceae is that their members are non-acid-fast. Thus, the cultivation of *E. coli* occurs at pH 7.2–7.5, *K. pneumoniae* – at 7.2. Enterohemorrhagic *Escherichia coli* can grow in acidic environments up to pH 4.4 [7]. Therefore, in addition to competition for plastic material to carry out their activities, a restraining factor for the growth of commensal *E. coli* may be a low redox potential of the intestine, due to the products of life of opportunistic pathogens. Quantitative reduction of commensal *E. coli* in children with colic may be an evidence not only of competition with other microorganisms for nutrients, but also a decrease in the intestinal pH to levels that inhibit the reproduction and growth of *Escherichia coli*.

Obviously, low values of redox-intestinal potential, which occur during the life of pathological flora, can have an inhibitory effect on some microorganisms and promote the growth of others, and disrupt the digestive process. Scientific studies have shown that the fluids secreted into the intestine are alkaline: pH of the small intestine fluid = 7.5–8.0, pH of the secretion of the colon – 7.5–8.0, the secretion of the Bruner glands – 8.0–8.9 [10]. This determines the correct digestive processes. Changing the pH of the intestine to the acidic side leads to a violation of digestive processes with all the ensuing consequences: flatulence, spasms of the intestine, pain, crying, irritability of the child, etc.

Evidence of a decrease in redox potential of the intestine is the appearance or increase in the concentration of opportunistic flora, especially fungal (*Candida*), which grows in a neutral, slightly acidic or acidic environment [4], members of the Enterobacteriaceae and Enterococcaceae families due to acid metabolism and/or gaseous products.

Representatives of the genus *Klebsiella*, which contaminated the intestine in more than a third of patients (22–39 %), were *K. pneumoniae* – 20 (35 %), *K. oxytoca* – 2 (3 %). There was a positive correlation between *K. pneumoniae* and fungi of the genus *Candida* ($r=0.48$, $p=0.0098$). Representatives of the genus *Klebsiella* have a significant potential for pathogenicity: persistent, haemolytic and adhesive activity, is a producer of DNA-bases, suppress phagocytosis [1].

Conclusion

Children with intestinal colic have disorders in the gut microbiota – expansion of facultative anaerobic bacteria (such as Proteobacteria), mainly members of the family Enterobacteriaceae, increased number of microorganisms such as Firmicutes, family Staphylococcaceae, fungi of the genus *Candida*, decrease in obligate anaerobic bacteria.

Indirect evidence of the shift of redox – the potential of the intestine in the acidic direction are: the appearance of representatives of opportunistic flora, which cultivation occurs at neutral or low redox potential of the intestine (fungi of the genus *Candida*, ENES); reducing the number of bacterial representatives, which products are organic acids (commensal *E. coli*, Lacto- and Bifidumflora).

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