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Intestinal colonization in Polish infants: From newborns till 18-month-old children

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ABSTRACT

Introduction: Human intestinal colonization is a dynamic process that is nowadays redefining due to hygienic changes in the Polish population.

Aim: To analyze the development of the intestinal flora from newborns till 18-month-old infants in Poland.

Material and methods: 171 newborns were enrolled. We collected fecal samples at 5 timepoints (1st stool, at 3, 6, 12, 18 months). At each visit, the questionnaire concerning breastfeeding, antibiotics, probiotics was obtained including atopy family history at the first visit.

Results: The count of staphylococci, enterococci, lactobacilli decreased (mean 0 months vs. 18 months: 3.08×10^7 CFU/g vs. 6.35×10^6 CFU/g; 1.85×10^{10} CFU/g vs. 9.26×10^7 CFU/g; 3.3×10^{11} CFU/g vs. 3.11×10^7 CFU/g) and *Clostridium difficile* and Gram-negative bacilli increased (6.2×10^4 CFU/g vs. 1.34×10^5 CFU/g; 1.78×10^6 CFU/g vs. 9.03×10^7 CFU/g) during the first 18 months of life. Positive maternal atopy history influenced colonization with staphylococci in newborns, anaerobic bacteria, enterococci in 3-month-old infants and anaerobic bacteria in 6-month-old infants.

Discussion: Our study shows that the gut colonization is a constant process. For the first time, we present the trends in bacterial establishment in a group of more than 170 Polish children.

The positive role of breastfeeding in the establishment of gut flora was previously suggested. Unexpectedly, among mostly breastfed children no relation between breastfeeding and the infantile gut microflora was found.

Conclusions: The intestinal colonization is continuously changed over the first 18 months of life and is influenced by positive maternal atopy history.

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1. Introduction

The human adult gastrointestinal microflora is, under normal circumstances, a stable ecosystem. The opposite situation is observed during the postnatal period, when intestinal microflora is a subject for constant changes.

For over 20 years scientists were trying to follow up its establishment and the factors influencing its composition in early infancy.[1,2] The background for these studies was that bacteria in human gut may induce long-term consequences for the child's health[3] and a single pathogen may become a source of infection, especially in preterm infants.[4]

Bacteria colonize the intestinal tract immediately after delivery, firstly predominated by *Escherichia coli* and enterococci and later enriched with anaerobic bacteria such as bifidobacteria, clostridia and *Bacteroides*.[5] Other anaerobic bacteria are successively acquired, creating a highly diverse microflora found in older children.[6] Yet, the process of colonization is complicated and it might be influenced by a bunch of different factors as the hospital environment during the delivery, prematurity, hygiene habits in the population, type of infants feeding,[7] antibiotics application or probiotics substitution.[8] The environmental sources seem to be very important because the differences in the colonization pattern were previously shown between developing and western societies e.g. Ethiopian–Swedish newborns[9] or Estonian and Swedish infants.[10]

2. Aim

The present study aimed to characterize the composition of fecal flora in the country that used to be encountered as developing country and in the last decade its population changed the nutritional and living habits, similarly to Western societies. We additionally assessed the role of breastfeeding and positive atopy family history in the establishment of intestinal flora in newborns and infants.

3. Material and methods

3.1. Patients

During a 3-year period at the Department of Pediatrics, Medical University of Silesia, 171 healthy newborns born vaginally were recruited to the present study. All enrolled newborns were discharged from the hospital at standard time after 72 h. In total, 120 18-month-old children finished the study. We have not obtained full 5 fecal samples from 51 infants due to inconsistent parental appearance during follow up. The inclusion criteria were: healthy, born at term newborns without any signs of infection (neither maternal infection or child's infection at perinatal period) and uncomplicated pregnancy. The parents were informed verbally and in writing regarding the nature and requirements of the study. Their written informed consent was obtained, and the study was approved by the Ethical Committee of Medical University of Silesia.

3.2. Clinical evaluation

During each visit the children were clinically examined by the same pediatrician and the questionnaire was filled out including information about family size, household, type of feeding, antibiotic treatment and oral probiotic supplementation. The study was designed to provide 4 pediatrician's visits (at age 3, 6, 12 and 18 months) with fecal samples collection. The enrollment in our study included muster of the first newborn's feces for microbiologic analysis and establishment of atopy family history for each newborn.

3.3. Fecal bacterial analysis

Approximately 1 g voided stool was collected into sterile plastic containers by the parents (1st stool after delivery was taken by qualified nurse at the delivery room) at the day of pediatrician's visit. All samples were kept at −20°C for not more than 3 months.

All fecal samples were cultured on Schaedler Agar, sequentially diluted (from 10^{-2} to 10^{-9}) and inoculated on selective media (BioMerieux, Warsaw, PL): yeasts and fungi on Sabouraud medium with chloramphenicol as described above; aerobic bacteria were cultured on MacConkey agar; Chapman medium and D-Coccosel agar.

Anaerobic bacteria were incubated for 4–5 days in anaerobic condition at temp. 37°C in GENbox anaer (BioMerieux, Warsaw, PL). Anaerobes were cultured on fastidious anaerobe agar (FAA) (LAB M, Lancs, UK), anaerobic gram-positive bacteria on Columbia CNA Agar (BioMerieux, Warsaw, PL), anaerobic gram-negative bacteria on Schaedler'a Neo. Vanco agar (BioMerieux, Warsaw, PL), *Clostridium difficile* on clostridium difficile agar (BioMerieux, Warsaw, PL). Total count of clostridia was estimated after 1-h-incubation of equal portion of feces and ethyl alcohol, then diluted and inoculated on FAA. Lactobacilli were cultured in anaerobic condition at temperature 37°C for 72 h on Rogosa agar (DIFCO, Plymouth, UK). The detection limit of microorganisms was 3 log CFU g^{-1}.[10]

3.4. Statistical methods

Statistical analyses regarding overall bacterial colonization rate were performed for every feces' collection point (newborns, 3, 6, 12, 18 months) separately. To observe the changes in the colonization rate in relation to time we used Kruskal–Wallis test. Children given antibiotic treatment during the follow-up period were divided according to the route of antibiotic application: oral use (OU), intra-muscular injection (IMI) or both. These children and children who did not receive any antibiotic treatment were compared using Kruskal–Wallis test. Finally, we tested the general hypothesis of antibiotic influence on infantile intestinal flora based on the answer 'yes' for antibiotics used or 'no' for no antibiotic treatment during the observation period using Mann–Whitney *U* test. Breastfeeding as factor driving the development of intestinal flora was analyzed using Kruskal–Wallis test.

When recruited, children were classified into one of two subgroups depending on maternal atopy history. We categorized children into maternal atopy positive group when mother was suffering from atopic eczema, allergic rhinitis

or asthma and the disease was clinically proven. Group 1 (no maternal history of atopy) consisted of 150 children and group 2 (children with positive maternal atopy history) included 21 children.

Allergic diseases were diagnosed as follows: (1) asthma was defined as three or more episodes of bronchial obstruction/wheezing, at least once verified by a physician or two episodes of bronchial obstruction with eczema or food allergy; (2) allergic rhino-conjunctivitis was defined as rhinitis and conjunctivitis appearing at least twice after exposure to a particular allergen and not related to infection; (3) food-related gastrointestinal problems were defined as vomiting and/or diarrhea on at least two separate occasions after intake of certain offending food; (4) atopic dermatitis was defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution.

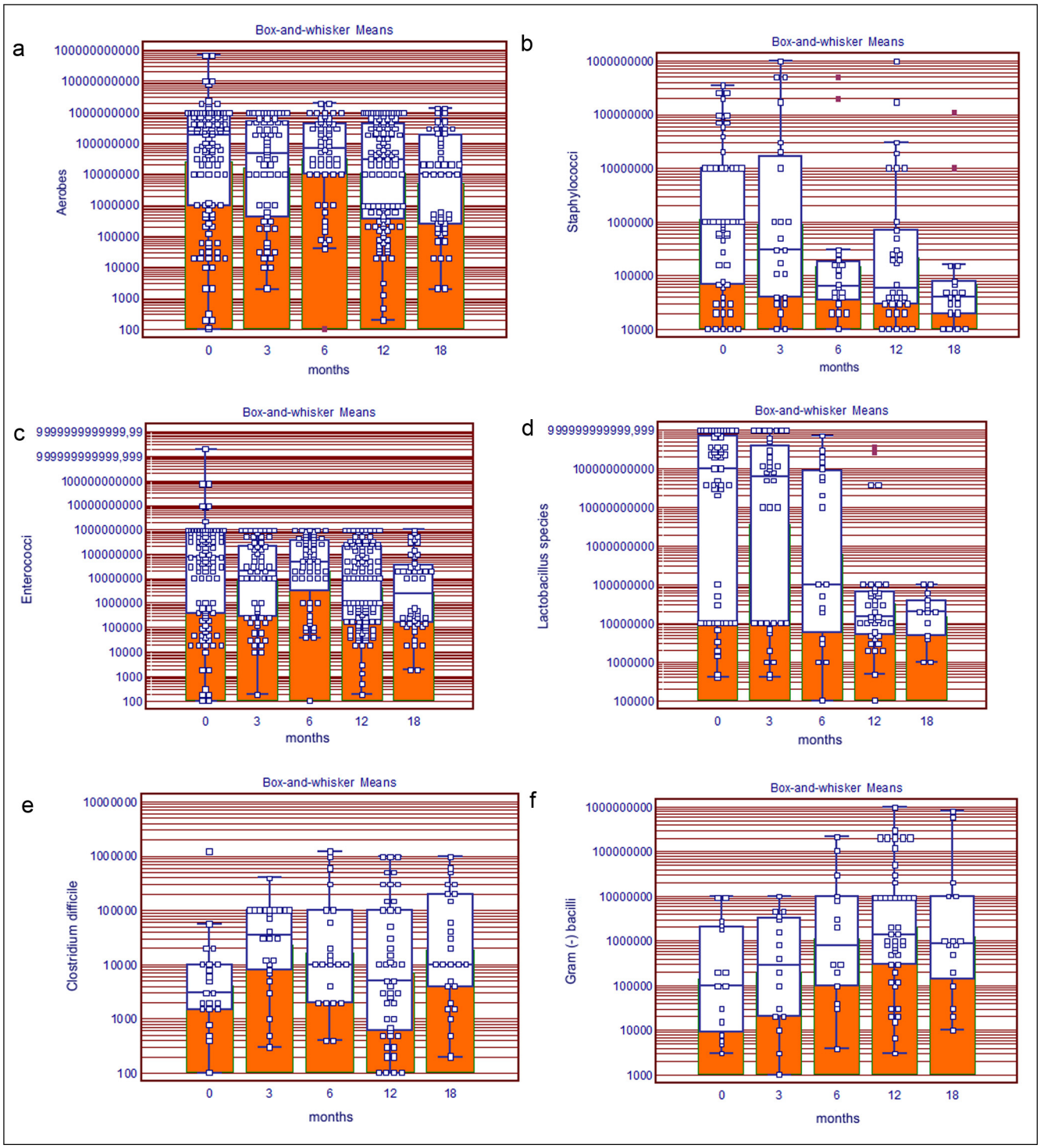

Fig. 1 – Changes in the count of (a) total aerobic bacteria; (b) staphylococci; (c) enterococci; (d) lactobacilli; (e) *Clostridium difficile*; (f) gram-negative bacilli in the first 18 months of life.

4. Results

4.1. Population levels of intestinal bacteria

We analyzed the overall counts of total anaerobes, *E. coli*, staphylococci, enterococci, total anaerobes, clostridia, *C. difficile*, Gram-negative bacilli, lactobacilli and *Candida* spp. We detected a statistically significant decrease in the count of (1) total aerobes ($P = 0.02$) (Fig. 1a), (2) staphylococci ($P = 0.00$) (Fig. 1b and c) enterococci ($P = 0.00$) (Fig. 1c) and (4) lactobacilli ($P = 0.00$) (Fig. 1d) during the first 18 months. On the other hand, the count of (5) *C. difficile* ($P = 0.03$) (Fig. 1e) and (6) Gram-negative bacilli ($P = 0.03$) (Fig. 1f) increased with age.

4.2. Antibiotics, probiotics and intestinal colonization

During the observation period, the children if necessary received the antibiotic treatment. The study protocol allowed treating with sulfametazone and trimetoprim, penicillin, cefalexin. Regarding the route of administration, children were given antibiotics either orally, intramuscularly or intravenously. In Table 1, we presented the number of children that received antibiotics through one of administration routes. Infants treated with antibiotics over the period of study had lower number of fecal lactobacilli at the end of study comparing to the non-treated children ($P = 0.04$) (Fig. 2). Fig. 2 presents number of *Lactobacillus* bacteria in group of non-treated children and number of *Lactobacillus* bacteria in group 1 (children who received antibiotics more than once during the study period). We did not analyze if more than once antibiotic administration changed the lactobacilli count at the infants' stool. Our study did not reveal the significant difference in the number of other aerobic or anaerobic bacteria.

In the studied group, 17 infants had a probiotic drug taken once (Lakcid, Biomed, Lublin, PL), 7 infants were supplemented twice in the follow-up, and 2 children more than twice. The total group was divided into 3 subgroups: 0–6 months old (subgroup A), 6–12 months old (subgroup B), 12–18 months old (subgroup C). In the individual analysis for every child it appeared that in the group A 2 out of 4 children had lactobacilli (50%), in the group B 2 out of 4 children (50%), group C 9 out of 14 children (64%), and lastly in the group D 1 child out of 4 (25%). However due to lack of molecular identifications of strains and genus, our findings cannot support the real effect of probiotics in our studied group.

4.3. Breastfeeding and intestinal colonization

Most infants ($n = 80$) were breastfed exclusively up to the first 3 months of life, whereas 56 children were breastfed till 6 months, 28 children till 12 months and 7 infants until 18 months. No impact of breastfeeding on certain bacteria was noted in our group.

Table 1 – Number of children receiving antibiotics through different routes of administration.

Antibiotic route	0–3 months	3–6 months	6–12 months	12–18 months
Oral	0	0	9	12
Intramuscular	3	3	0	1
Intravenous	1	0	1	1

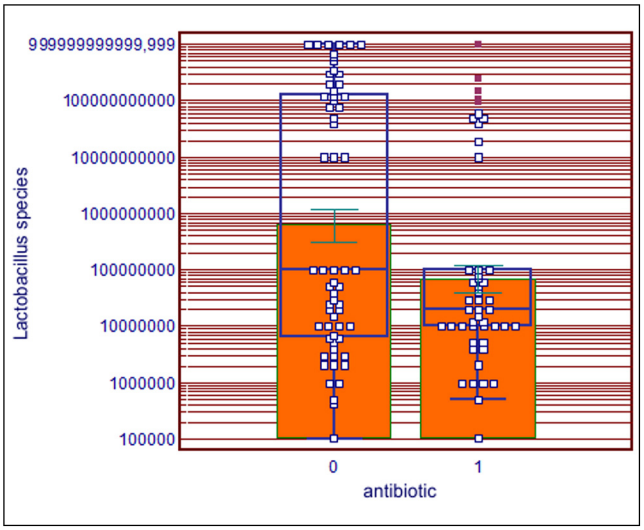

Fig. 2 – Lactobacilli in the stool of antibiotic-treated children vs. others.

4.4. Atopic background and intestinal colonization

In our study, positive maternal atopy history influenced colonization with: staphylococci in newborns ($P = 0.04$; Fig. 3a), anaerobic bacteria ($P = 0.02$; Fig. 3b), enterococci ($P = 0.02$; Fig. 3c) in 3-month-old infants and anaerobic bacteria ($P = 0.02$, Fig. 3d) in 6-month-old infants.

4.5. Atopic disease and intestinal colonization

Asthma was diagnosed in 4 children. Allergic rhino-conjunctivitis in 19 children. Food-related gastro-intestinal problems in 15 children and atopic dermatitis in 16 children. The children with food-related gastro-intestinal allergic disease had an increased amount of *C. difficile* at 3rd month of life, but we have not confirmed the influence of other bacteria on the development of allergic diseases during 18 months of study (asthma, gastro-intestinal allergic disease, allergic rhinitis, atopic dermatitis).

5. Discussion

Our study shows that the gut colonization is a constant process. For the first time, we present the trends in bacterial establishment in a group of more than 170 Polish children. In place of decreasing amounts of staphylococci, enterococci and lactobacilli, the increasing amount of *C. difficile* and gram-negative rods appears with time.

Vael et al. stated that the large scale epidemiological studies studying the microbial gut flora in young children has been lacking.[11] Therefore, we undertook the aim to evaluate the role of breastfeeding, antibiotic treatment and maternal

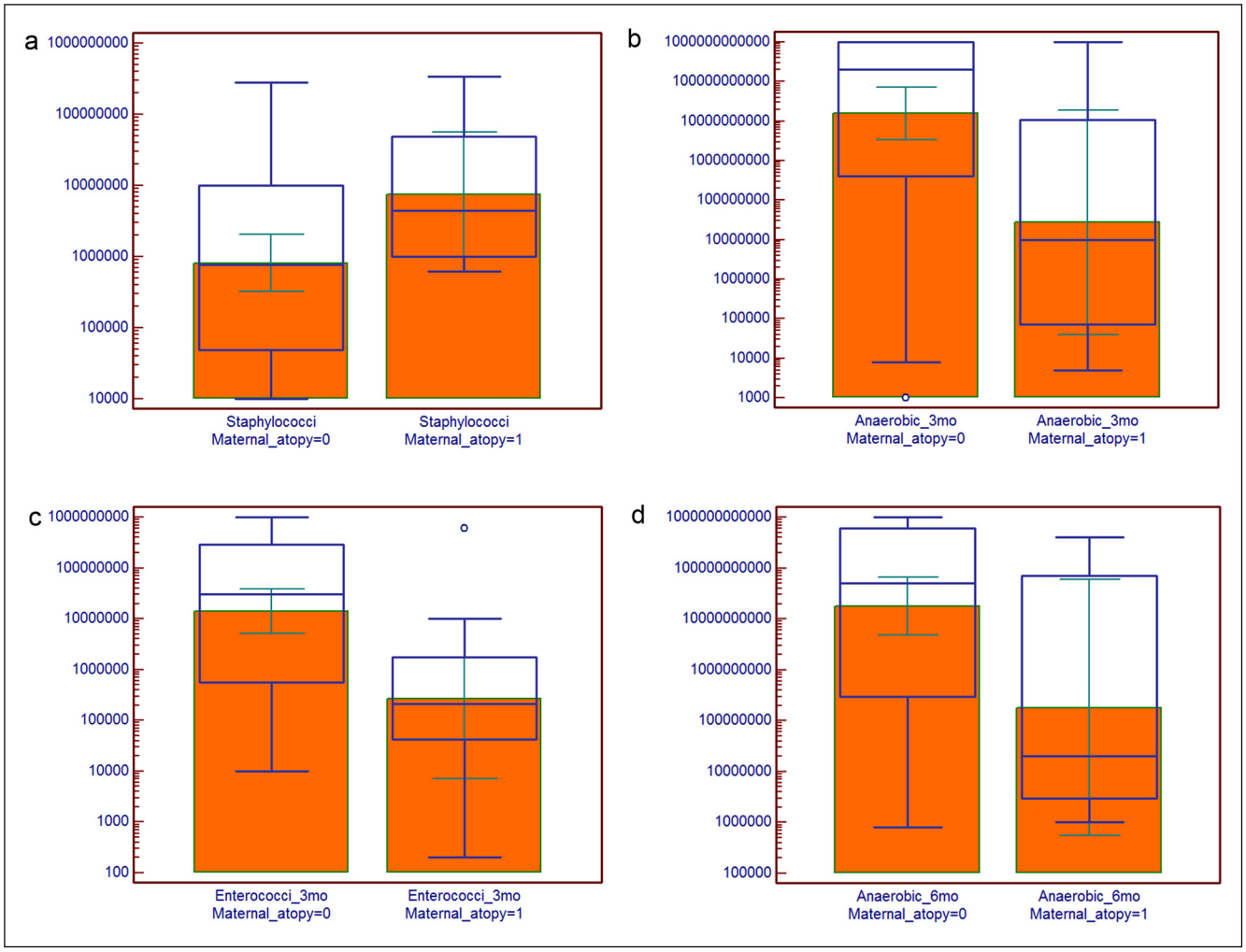

Fig. 3 – Bacteria colonization in atopic vs. non-atopic infants: (a) staphylococci in the 1st stool after delivery at newborns; (b) anaerobic bacteria in 3rd month; (c) enterococci at 3rd month; (d) anaerobic bacteria at 6th month.

family atopy history in the gut colonization of Polish infants. Some previous studies showed the differences between allergic and non-allergic infants such as an increased amount of *Clostridia* and decreased *Bifidobacteria* at 3 weeks and 3 months of age in children who had later developed atopic diseases.[12] In addition, early *Bacteroides fragilis* colonization at 3 weeks of age was linked to later asthma development.[11] We noted the increased levels of *C. difficile* at 3 months of age had been related to the development of allergic diseases in toddlers. It could be explained by the usage of less selective culture media than in the other studies, by shorter period of patient observation or by decreased importance of gut bacteria as factor influencing the development of allergic diseases in Polish population.

Taking into consideration that approximately 60%–70% of healthy newborns and infants are colonized with *C. difficile*,[13] the finding that *C. difficile* colonization in early infancy might contribute to the later development of allergic gastrointestinal symptoms, should have risen a special attention. The role of clostridia in allergy development was mentioned previously by Bjorksten et al.[3] Recently, *C. difficile* is reported to increase the risk of atopic eczema, recurrent wheeze and allergic sensitization.[14] Thus, our findings broaden the knowledge about the possible role of *C. difficile*. It should have been noted that in our study, comparing to study by Penders, we collect 5 fecal samples and the only significant implication was found in 3-month-old children as in study by Bjorksten.[3]

However, our general results seem to be resembling those showing that neither atopic eczema or food-specific IgE were associated with acquisition of certain bacterial groups in 3 European cohorts.[15]

The positive role of breastfeeding in the establishment of gut flora was previously suggested. We investigated whether breastfeeding had changed the composition of gut microflora because the maternal milk had been known as a source of bifidobacteria[16] and lactobacilli.[17] It is unexpected that among mostly breastfed children, we could not detect any linkage between breastfeeding and the infantile gut microflora. It could be explained by lack of bacterial tracking between mother and her child in our study. Due to similar reasons, we find it difficult to evaluate the real effectiveness of probiotics in

the studied group. We could not follow the passage of certain strains of lactobacilli in the infantile intestines.

However, we find it important that the positive atopy history – a genetic factor-shifts the intestinal colonization toward anaerobic bacteria, especially enterococci in the first months of life. It seems to be the first report showing the difference of intestinal colonization depending on maternal atopy in Polish children.

6. Conclusions

We present the first study among Polish newborns and infants concerning the gut microflora colonization in early infancy. Regarding the consecutive follow-up of children and stool collection, the study brings new insight into the bacterial colonization in the population with merging social changes including hygiene and eating habits. It should be underlined that mucus membrane of gastro-intestinal tract covers around 300 m^2 making it the largest surface of contact between organism and environmental factors such as micropathogens.[18]

Conflict of interest

None declared.

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