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New Perspective on chronic functional constipation in children with the identification of the microbiota

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Abstract

Chronic functional constipation is a common gastrointestinal disorder in children with no underlying organic cause. Although reasons such as bad eating habits and early transition to solid food are blamed for constipation, the etiology of the disease has not been fully elucidated. Our study aims to determine the differences in stool microbiota between children with chronic functional constipation and healthy children. Feces samples of 49 patients and 40 healthy children who meet Rome IV criteria were analyzed by 16s rRNA /PCR (Polymerase Chain Reaction) method. Within the sample microbial diversity, the Shannon diversity index was calculated based on the profiles obtained using the R 2.15.2 software package programe. In our study, no statistically significant difference was found between the study group and the control group in terms of the Shannon Diversity Index ($p < 0.05$). The species found in healthy children ($n = 34$) but not in constipated children were determined as Lactobacillus kefiri and Bifidobacterium infantis. In addition, Lactobacillus casei and Lactobacillus acidophilus were detected with a higher rate in constipated children ($p > 0.005$). Although there is no significant difference between microbiota subtypes, the fact that Lactobacillus kefiri and Bifidobacterium infantis were detected only in healthy children may guide the supportive treatments to be given to constipated children. The results of our study also show that there is a need for more comprehensive studies in large populations, supporting other literature studies showing that the gastrointestinal microbiota is different in constipated and normal children.

Keywords: Bifidobacterium infantis, child, constipation, microbiota, lactobacillus kefiri

Introduction

Chronic functional constipation is a common gastrointestinal disorder in children with no underlying organic cause. Although reasons such as bad eating habits and early transition to solid food are blamed for constipation, the etiology of the disease has not been fully elucidated. In patients; there is no known cure for constipation, except for behavioral-diet therapy and a few symptomatically laxative products [1]. Many patients do not respond to symptomatic treatment over time, and this chronic process prevents the absorption of especially fat-soluble vitamins and minerals with prolonged use of laxatives, and in this case, children experience anorexia, growth retardation, symptoms related to vitamin deficiencies, and many accompanying complications [2]. Therefore, elucidating the etiology of constipation is very valuable in revealing new treatment options. For this purpose, our study was planned to determine the microbiota differences in children with and without constipation.

In recent studies, the knowledge that bacteria constitute approximately 50% of the stool volume suggests that there may be a relationship between microbiota and functional chronic constipation [3]. Staphylococci, Streptococci, Lactobacillus, Micrococci, and Bifidobacteria, especially transmitted with breast milk, constitute the infant's first microbial flora [4]. Over time, this flora changes with many factors such as the baby's diet, history of antibiotic use, and illnesses. Bacteroides, Lactobacillus, and Saccharomyces species among the Bifidobacterium yeasts are the most common bacteria found in the intestine that has completed its maturation. Disruption of the balance in this microbial flora

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with various diseases or drug treatments may also play a role in the formation mechanism of different diseases. In other words, microbial balance is lost in patients in the form of a vicious circle and returns to its place in time or it is tried to be supplemented with supplementary probiotics [3,4]. There are also studies suggesting that intestinal cells directly affect our brain with their microbes and their functional structure and can play a key role in behavioral and cognitive functions [5]. In recent years, intestinal microbiota and brain axis issues have been seen as the key point to answer the question of "can they play a role in etiology?" For many diseases that we previously considered as functional and/or idiopathic [5].

The number of studies examining the relationship between regional childhood functional constipation and microbiota is very limited worldwide [6,7]. The reason for this can be stated as laborious studies, difficulties in taking samples from children, and high costs [4-7]. Our study was planned to determine the diversity of microbiota in chronic functional constipated children to reveal the difference from healthy children by the financial support of our university's scientific research project coordinator (Project no: TS-2019-04).

Materials and Methods

Before starting the study, the approval of the local ethics committee was obtained (2018/80576354-050-99/89) and a voluntary consent form was obtained from the patient and healthy children's parents before the samples were taken. In our study, stool samples to be used for the detection of the gastrointestinal microbiota, 49 constipated children aged 2-16 years who meet Rome 4 criteria and 40 healthy children who applied to the outpatient clinic for routine controls (weight, height, etc.) without acute / chronic infection/ disease were included. Children with a history of preterm birth, anatomical abnormalities, using antibiotics in the last 2 months, obesity or malnutrition, using medication due to chronic disease, and feeding with formula were excluded from the study. After the feces samples of the patients were taken, they were kept at -80ºC. Our work is supported by our university's scientific research project support fund (2019-TS04).

Genomic DNA isolation protocol

The samples were taken into an Eppendorf tube. Over; 200µl dH_2O, 50µl 0.5M EDTA, 10µl 20% sarcosyl, 10µl proteinase K (10mg / ml), 10µl 1M Tris-HCl (pH: 8) and 5µl 5M NaCl were added. The mixture was vortexed for 5 minutes. It was kept in a water bath set at 65°C for 30 minutes. During this period, it was vortexed every 10 minutes. Phenol: chloroform: isoamyl alcohol (25:24:1) in its volume was added to the cell suspension and gently inverted. It was centrifuged for 5 minutes at 13.000 rpm, the upper liquid layer was removed with a Pasteur pipette (or a 1000 µl micropipette whose mouth was cut with a razor blade) and transferred to a new tube. Phenol: chloroform: isoamyl alcohol treatment was carried out 3 times as stated above. The supernatant was taken from the products obtained at the end of centrifugation in each step and transferred to a clean Eppendorf tube. 1/10 of its volume 3M NaAc and 2 times its volume of absolute ethanol were added to the upper liquid, which was taken into the new Eppendorf, and it was kept at -20°C for 1 night. At the end of the period, the sample was centrifuged at 13.000 rpm for 10 minutes. The supernatant was removed, the pellet dried. 200µl dH_2O was added onto the pellet and the pellet dissolved. 1/10 volume of 0.3M NaOAc and 440µl ethanol were added onto the dissolved pellet and kept at -20°C for 1 night. At the end of the period, the sample was centrifuged at 13.000 rpm for 10 minutes. The supernatant was removed and the pellet was allowed to dry.

After drying, the pellet was dissolved in 100 µl dH_2O. Genomic DNA obtained; It was checked in terms of its quality, RNA contamination, and integrity, respectively, according to spectrophotometric measurement and its appearance in 0.8% agarose gel.

Determination of DNA Sequence Information

16S rRNA primers specific to bacteria and accepted as universal to determine DNA sequence data [Primer 1: 27 F (5'-GAG TTT GAT CCT GGC TCA-3 ') and Primer 2: PCR reaction was performed with (1385R) (5'-CGGTGTGT [A/G] CAAGGCCC-3')]. Genomic DNA was used as source DNA in the PCR reaction. The PCR reaction was set up with 16S rRNA (F and R) primers; 2.5 µl 10X buffer, 2.5 µl 25 mM $MgCl_2$, 2 µl 2.5 µM dNTP mixture, 2.5 µl F, 2.5 µl R, 0.5 µl genomic DNA, 0.2 µl Taq DNA Polymerase enzyme (5u / µl) with a final volume of 25 µl 12.3 µl ddH_2O was added. PCR program used for both products: 2 minutes at 94ºC, 1 minute at 94ºC, 1 minute at 55ºC, 1 minute at 72ºC, 4 minutes at 72ºC. and it was used to be at 4ºC. DNA fragments run in agarose gel were checked on the UVP transilluminator device and the data was recorded with the UV-Photometer gel documentation device (UviTec).

Microbiota Analysis

The total DNA obtained was separated in 110 µl buffer and used for PCR amplification. ISpro technique: Isolated DNA (10µl / PCR) was amplified in two multiplex PCR amplifications: first; It was made for Firmicutes, Actinobacteria, Fusobacteria, Verrucomicrobia (FAFV), Bacteroidetes species. Latter; Made for Proteobacteria species. After amplification, 5 µl of PCR product was mixed with 20 µl formamide and 0.2 µl Mapmaker 1500 ROX labeled size marker. The PCR products were then separated by their different lengths in an ABI Prism 3130XL Genetic Fragment Analyzer.

Statistical analyses

Data were analyzed with the standard IS-pro proprietary software package. A correlation matrix of all log2 transformed profile data was generated. A clustered heat map was made with the arithmetic mean (UPGMA). Within the sample microbial diversity, the Shannon diversity index was calculated based on the profiles obtained using the R 2.15.2 software package. Diversity was calculated for both per phylum and overall microbial composition (phylum FAFV, Bacteroidetes, and Proteobacteria were pooled together). A p-value of <0.05 was considered statistically significant. Differences in microbiota composition were shown as basic coordinate analysis (PCoA) based on cosine distance measurements. Data visualizations were made with the Spotfire software package.

Results

The study was carried out with 49 patients and 40 healthy children. The demographic characteristics of the patients included in the

study are shown in Table 1.

The 16S RNA gene was obtained by PCR. PCR results of sick individuals are shown in Figure 1, PCR results of healthy individuals are shown in Figure 2.

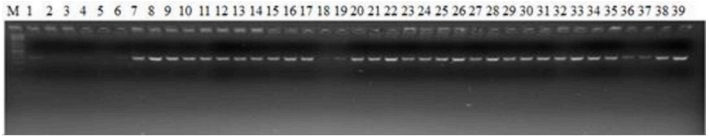

Figure 1. Image of PCR results of sick individuals

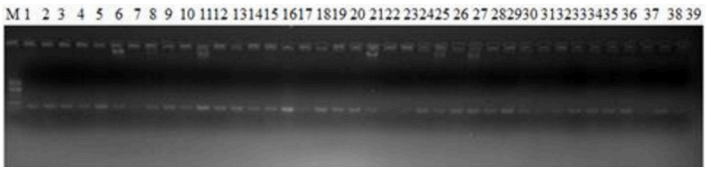

Figure 2. Image of PCR results of healthy individuals

Table 1. Demographic characteristics of the patients, evaluation of the clinical severity of constipation

	Patients	Healthy	p
Age (years)	**6.03±3.81**	**6.5±4.12**	**0.701**
Male/Female (%)	46/54	48/52	0.886
Number of stools per week			
<1	24	-	
1-2	15		
>2	10		
The need for stimuli while defecation	17	-	
Tenesmus	12	-	
Abdominal pain	14	-	
A history of stool incontinence	**9**	**-**	
Benefit from laxatives	8	-	
Bristol stool scale 1/2/3	22/19/8	--	
Benefit rate from nutritional regulation	13		
Nutrition and dietary habits			
Protein-based	15	18	0.325
Vegetable and fiber-based	2	1	
Irregular, fast food	9	5	
Balanced diet	23	25	

Microbiota Analysis

Consistent associations of constipation microbiota properties with diseases have been established. Both diseases and microbiota properties were clustered according to the cosine distances generated from the beta coefficients of all nominally significant ($p<0.05$) relationships. Beta coefficients were converted to arcine for viewing.

Non-significant relationships were scored in 0 and therefore white. Diseases or microbiota properties with no significant association were not shown. The bootstrap clustering of microbiome features, highlighted in the left dendrogram, were identified in two study groups.

The most abundant species in IS profiles of both study groups are Bifidobacterium lactis; Bifidobacterium longum, Bifidobacteria bifidus, Bifidobacterium infantis, Bifidobacterium lactis; Bifidobacterium longum, Bifidobacteria bifidus, Bifidobacterium infantis, Bifidobacterium bifidum, Lactobacillus agilis, Lactobacillus animalis, Lactobacillus branta, Lactobacillus brevis, Lactobacillus camelliae, Lactobacillus paracasei, Lactobacillus catenefornis Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus namurensis, Lactobacillus Plantarum, Lactobacillus rhamnosus, Lactobacillus reuteri Lactobacillus similis, Lactobacillus thailandensis, S. aureus, S. epidermidis, S. haemolyticus, Saccharomyces boulardii (Figure 3).

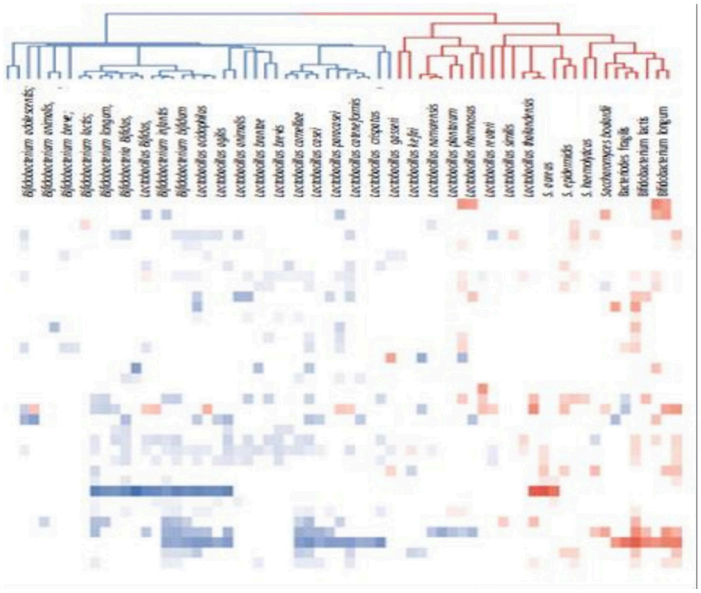

Figure 3. Microbiota clusters image in sick and healthy individuals

The cluster map showed disease-specific clustering at both the phylum level and the species level. Although it is much more intense especially in healthy individuals numbered 6 and 18, the species in the vast majority of healthy individuals (n=34) but never detected in sick individuals are Lactobacillus kefiri and Bifidobacterium infantis. In addition, the species identified in patients with numbers 2, 7, 13, 26, 39 but not in healthy individuals are Lactobacillus bifidus, Lactobacillus acidophilus, Lactobacillus rhamnosus.

In our study, the Shannon diversity index did not show a statistically significant difference between the study group and the control group in terms of phylum-level diversity. Apart from that, PCoA distinguishes between children with functional constipation and healthy controls, both for all phyla and each phylum. In our study, values showing a similar diversity index for all phyla were combined and children with functional constipation were determined as 3.2 (IQR 0.3) and controls as 3.1 (IQR 0.3). Species determined in sick individuals as a result of microbiota analysis are shown in Table 2 and species determined in healthy individuals are shown in Table 3.

Table 2. Microbiota results of sick individuals

Patient no	Microbiome species
1, 2 ,3, 4, 5, 6, 8, 12, 13, 14, 15, 16, 17, 18, 19, 21, 25, 26, 28, 30, 31, 46, 47	Bacteroides fragilis
	Bifidobacterium lactis
	Bifidobacterium longum,
	Lactobacillus Bifidus
	Lactobacillus acidophilus
	Lactobacillus reuteri,
	Lactobacillus rhamnosus
	Bifidobacterium adolescentis;
	Bifidobacterium animalis,
	Bifidobacterium breve
7,9, 10, 11, 20, 22, 23, 24, 27, 29, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41 42, 43, 44, 45, 48, 49	Bifidobacterium adolescentis;
	Bifidobacterium animalis,
	Bifidobacterium breve;
	Bifidobacterium lactis;
	Bifidobacterium longum,
	Bifidobacteria Bifidus,
	Lactobacillus Bifidus
	Bifidobacterium infantis
	Bifidobacterium bifidum
	Lactobacillus acidophilus*
	Lactobacillus agilis
	Lactobacillus animalis
	Lactobacillus brane
	Lactobacillus Brevis
	Lactobacillus camelliae
	Lactobacillus casei
	Lactobacillus paracasei
	Lactobacillus catenefornis
	Lactobacillus crispatus
	Lactobacillus gasseri
	Lactobacillus namurensis
	Lactobacillus Plantarum
	Lactobacillus rhamnosus*
	Lactobacillus reuteri*
	Lactobacillus similis
	Lactobacillus thailandensis
	S. aureus
	S. epidermidis
	S. haemolyticus
	Saccharomyces boulardii

*:not found in healthy individuals

Table 3. Microbiota results of healthy individuals

Patient no	Microbiome species
8, 12, 13, 19, 21	Bifidobacterium adolescentis;
	Bifidobacterium animalis,
	Bifidobacterium breve;
	Bacteriodes fragilis
	Bifidobacterium longum,
	Lactobacillus Bifidus
	Lactobacillus rhamnosus,
1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 14, 15, 16, 17, 18, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34,3 5, 36, 37, 38, 39	Bifidobacterium lactis;
	Bifidobacterium longum,
	Bifidobacteria Bifidus,
	Bifidobacterium infantis*
	Bifidobacterium bifidum
	Lactobacillus agilis
	Lactobacillus animalis
	Lactobacillus brantae
	Lactobacillus Brevis
	Lactobacillus camelliae
	Lactobacillus paracasei
	Lactobacillus catenefornis
	Lactobacillus crispatus
	Lactobacillus gasseri
	Lactobacillus kefirii*
	Lactobacillus namurensis
	Lactobacillus Plantarum
	Lactobacillus rhamnosus
	Lactobacillus reuteri
	Lactobacillus similis
	Lactobacillus thailandensis
	S. aureus
	S. epidermidis
	S. haemolyticus
	Saccharomyces boulardii

*:not found in sick indivuals

Discussion

In this study, although the Shannon diversity index did not show a statistically significant difference between the study group and the control group in terms of phylum-level diversity, Lactobacillus kefiri, and Bifidobacterium infantis were found in the vast majority of healthy individuals (n = 34) and never detected in sick individuals. In addition, Lactobacillus casei and Lactobacillus acidophilus were detected at a higher rate in sick individuals. ($p > 0.005$)

Some studies show that gut microbiota directly affects gastrointestinal motility [8]. A study in rats shows that Lactobacillus and Bifidobacteria species shorten the intestinal emptying time and increase intestinal myoelectric activity [9]. In addition to animal experiments, in vitro demonstrating that some species such as Lactobacillus rhamnosus increase motility and muscle cell contractility in human intestinal cells, an important step has been taken in the microbiota-constipation relationship, but clear evidence has not been obtained in clinical studies [10]. The number of studies conducted in childhood is also relatively low. In some studies, differences were observed between microbial subtypes in children with chronic functional constriction, and some studies did not find a significant difference [9-11]. In a study conducted on the subject, stool samples of 28 chronic constipation and 14 healthy children were examined, and it was found that Clostridium and Bifidobacteria species were more in constipated patients [11]. On the other hand, Zhu et al. In their study in 2014, while there was no difference between Lactobacillus and Bifidobacterium species, they found a significant decrease in Prevetolla subspecies of Bacteroides species and a significant increase in Firmicutes species in constipated children [12]. In the study conducted by Meij et al. In 2016, they observed that although there was no statistical difference, some species such as Bacteroides fragilis, Bacteroides ovatus, Bifidobacterium longum, Parabacteroides were high, and Alistipes finegoldii species had less presence in the microbiota in constipated children [6]. However, Moraes et al. ın the study conducted in Brazil in 2016, Lactobacillus species were found less in constipated children, while no significant difference was found between Bifidobacterium species [7]. In this context, in our study, it is an important point to detect Lactobacillus kefiri and Bifidobacterium infantis only in healthy children. The main goal of all studies on the subject; if there is a deficiency of microbiota element in constipated children, it is a matter of wonder whether this situation can be eliminated with supportive treatments. Many of these studies, including our study, show that prebiotics or probiotics have important roles in the treatment of patients with constipation.

Probiotic agents developed as a result of various studies have begun to be used in the treatment of functional diarrhea and irritable bowel syndrome, which is the opposite situation [13,14]. Also, microbiota supplements and fecal transplantations applied to patients with severe necrotic enteritis and pseudomembranous enteritis have been life-saving [15, 16]. All these situations show that supportive treatments given to patients in cases such as diarrhea or constipation have positive effects on the flora [1,5, 8-13].

Studies are showing that Lactobacillus reuteri, Lactobacillus casei, which are among the Lactobacillus subspecies, are beneficial in chronic functional constipation [17-19]. However, in a study on Lactobacillus kefiri in mice, it was emphasized that it reduced pro-inflammatory mediators and could be an important supplement for inflammatory bowel diseases [20]. On the other hand, it has been found that kefiri drink may have positive effects in adult constipated patients, but microbiota analysis has not been performed [21]. However, no intervention study was conducted on Lactobacillus kefiri supplementation in constipated children, and again, no study was found in the literature to determine its relationship with constipated children. Our study differs in this respect and shows that a new probiotic supplement can be created should be supported by future studies. While Bifidobacterium infantis was found effective in irritable bowel syndrome in probiotic studies; Its efficacy in constipated children has not been demonstrated [22]. However, the number of patient populations in the studies was small and the data were evaluated as limited. In probiotic intervention studies with Lactobacillus reuteri, acidophilus, Casei, and Rhamnosus, no sufficient evidence has yet been revealed in terms of their effectiveness in relieving constipation, and the effect of these probiotic mixtures against functional constipation is another subject of Research [23,24].

Conclusion

Our study is among the first studies to determine the difference in intestinal microbiota in constipated children compared to children with normal defecation, which has not yet been done in our region and many cities. According to the results of our study, the detection of Lactobacillus kefirii and Bifidobacterium infantis in healthy individuals, and the detection of L. casei and L. acidophilus in sick individuals in children who do not have a significant difference in nutrition and diet in the patient and healthy group are very valuable in terms of drug potential.

Conflict of interests

The authors declare that there is no conflict of interest in the study.

Financial Disclosure

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Ethical approval

Before starting the study, the approval of the local ethics committee was obtained (2018 / 80576354-050-99 / 89).

References

1. Rajindrajith S, Devanarayana NM, Weerasooriya L, et al. Quality of life and somatic symptoms in children with constipation: a school-based study. J Pediatr. 2013;163:1069-72.
2. Madani S, Tsang L, Kamat D. Constipation in children: A practical review. Pediatr Ann. 2016;45:189-96.
3. Yağcı RV, Arslan N. Intestinal microbiota and its relation with nutrition. Turkiye Klinikleri J Pediatr Sci. 2012;8:12-8.
4. Matamoros S, Gras-Leguen C, Le Vacon F, et al. Development of intestinal microbiota in infants and its impact on health. Trends Microbiol. 2013;21:167-73.
5. Wang HX, Wang YP. Gut microbiota-brain axis. Chin Med J (Engl). 2016;129:2373-80.
6. Meij TGJ, de Groot EFJ, Eck A, et al. Characterization of microbiota in children with chronic functional constipation. PLoS ONE. 2016;11:e0164731.
7. Moraes JG, Farias ME, Motta A, et al. Fecal microbiota and diet of children with chronic constipation. Int J Pediatrics. 2016;2016:1-8.
8. Barbara G, Stanghellini V, Brandi G, et al. Interactions between commensal bacteria and gut sensorimotor function in health and disease. Am J Gastroenterol. 2005;100:2560-8.
9. Husebye E, Hellström PM, Sundler F, et al. Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats. Am J Physiol Gastrointest Liver Physiol. 2001;280:368-80.
10. Guarino MP, Altomare A, Stasi E, et al. Effect of acute mucosal exposure to Lactobacillus rhamnosus GG on human colonic smooth muscle cells. J Clin Gastroenterol. 2008;42:185-90.
11. Zoppi G, Cinquetti M, Luciano A, et al. The intestinal ecosystem in chronic functional constipation. Acta Paediatr. 1998;87:836-41.

12. Zhu L, Liu W, Alkhouri R, et al. Structural changes in the gut microbiome of constipated patients. Physiol Genomics. 2014;46:679-86.

13. 13. Bhattarai Y, Muniz Pedrogo DA, Kashyap PC. Irritable bowel syndrome: a gut microbiota-related disorder? Am J Physiol Gastrointest Liver Physiol. 2017;312:52-62.

14. Lai HH, Chiu CH, Kong MS, et al. Probiotic Lactobacillus casei: Effective for managing childhood diarrhea by altering gut microbiota and attenuating fecal inflammatory markers. Nutrients. 2019;11:E1150.

15. Imdad A, Nicholson MR, Tanner-Smith EE, et al. Fecal transplantation for treatment of inflammatory bowel disease. Cochrane Database Syst Rev. 2018;11:1465-858.

16. Dave M, Higgins PD, Middha S, Rioux KP. The human gut microbiome: current knowledge, challenges, and future directions. Transl Res. 2012;160:246-57.

17. Tabbers MM, Chmielewska A, Roseboom MG, et al. Fermented milk containing Bifidobacterium lactis DN-173 010 in childhood constipation: a randomized, double-blind, controlled trial. Pediatr. 2011;127:1392-99.

18. Mazlyn MM, Nagarajah LH, Fatimah A, et al. Effects of probiotic fermented milk on functional constipation: a randomized, double-blind, placebo-controlled study. J Gastroenterol Hepatol. 2013;28:1141-7.

19. Coccorullo P, Strisciuglio C, Martinelli M, et al. Lactobacillus reuteri (DSM 17938) in infants with functional chronic constipation: a double-blind, randomized, placebo-controlled study. J Pediatr. 2010;157:598-602.

20. Carasi P, Racedo SM, Jacquot C, et al. Impact of kefir derived Lactobacillus kefiri on the mucosal immune response and gut microbiota. J Immunol Res. 2015;361604.

21. Turan İ, Dedeli O, Bor S, İlter T. Effects of a kefiri supplement on symptoms, colonic transit, and bowel satisfaction score in patients with chronic constipation: a pilot study. Turk J Gastroenterol. 2014;25:650-6.

22. Giannetti E, Maglione M, Alessandrella A, et al. A mixture of 3 bifidobacteria decrease the abdominal pain and improves the quality of life in children with irritable bowel syndrome: a multicenter, randomized, double-blind, placebo-controlled, crossover trial. J Clin Gastroenterol. 2017;51:5-10.

23. Wojtyniak K, Szajewska H. Systematic review: probiotics for functional constipation in children. Eur J Pediatr. 2017;176:1155-62.

24. Wojtyniak K, Horvath A, Dziechciarz P, et al. Lactobacillus casei rhamnosus Lcr35 in the management of functional constipation in children: a randomized trial. J Pediatr. 2017;184:101-5.