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Molecular assessment of fecal *lactobacilli* populations in children with functional constipation

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ABSTRACT – Background – Investigation of the gut-specific bacterial strains including lactobacilli is essential for understanding the bacterial etiology of constipation. **Objective –** This study aimed to compare the prevalence and quantity of intestinal lactobacilli in constipated children and healthy controls. **Methods –** Forty children fulfilling Rome IV criteria for functional constipation and 40 healthy controls were recruited. Fecal samples were analyzed using species-specific polymerase chain reaction followed by random amplified polymorphic DNA-PCR and quantitative real-time PCR. **Results –** Totally, seven different species of lactobacilli were detected. Out of 80 volunteers, 65 (81.3%) were culture and species-specific PCR positive from which 25 (38.46%) constipated children and 40 (61.54%) healthy subjects. The most prevalent species were *L. paracasei* 21 (32.3%) followed by *L. plantarum* 18 (27.7%) among both healthy and patient groups. Analysis of the RAPD dendrograms displayed that strains isolated from constipated and non-constipated children have similarity coefficients of more than 90%. The qPCR assays demonstrated constipated children had a lower amount of total lactobacilli population (per gram of feces) than healthy controls. **Conclusion –** Our findings showed that the mere existence of various species of *Lactobacillus* in the gut does not enough to prevent some gastrointestinal disorders such as functional constipation, and their quantity plays a more important role.

Keywords – Constipation; feces; *Lactobacillus*; prevalence.

INTRODUCTION

Functional constipation is a common and annoying problem in children with an estimated prevalence of around 3% worldwide. Functional constipation is often associated with infrequent bowel movements, fecal incontinence, painful defecation, and abdominal pain[1]. Currently, the diagnosis of this functional gastrointestinal disorder is performed using Rome IV diagnostic criteria[2]. The prevalence of constipation in children ranges from 0.7% to 29.6%, which constitutes 3% of the pediatric clinicians' visits and 30% of children's gastroenterologist referrals[3]. Although constipation may possess a variety of etiologies, alterations in gut flora, comprising the increase or decline of some species of the colonic microbiota, have been considered as possible pathogenesis of functional constipation in pediatrics[4]. To evaluate which probiotic strain may be useful in therapeutic strategies for functional constipation, a detailed description of the gut microbiota composition is crucial[5]. So far, probiotics and particularly *Lactobacillus* species have been used in several randomized controlled trials to treat various gastrointestinal disorders, including functional constipation[6]. However, given the paucity of data, it is unclear which probiotic species is most effective in the treatment of functional constipation[7,8]. The objective of this research was therefore to assess the prevalence, genetic heterogeneity and total amount of lactobacilli population in constipated children's fecal samples as compared to healthy controls utilizing species-specific polymerase chain reaction (PCR), randomly amplified polymorphic DNA-PCR (RAPD-PCR), and quantitative real-time PCR (qPCR).

METHODS

Subjects and study design

In this case-control study, we recruited 40 constipated patients from the tertiary pediatric gastroenterology department of the Abuzar children's hospital in Ahvaz between August 2018 and July 2019. The inclusion criteria were as follows: aged between 4 and 18 years and fulfilling diagnostic criteria for functional constipation based on the Rome IV definitions, which were examined and approved by a pediatrician. Participants with other metabolic and gastrointestinal diseases, a history of intestinal surgery, a history of receiving antibiotics within four weeks before enrollment, intake of oral laxatives four weeks ago, neurologic diseases (such as Hirschsprung's disease), mental retardation, and consumption of probiotics within 14 days before enrollment were excluded. The

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procedure of the study was explained to all participants and written consent was obtained from the children's parents. Additionally, 40 healthy subjects without any gastroenterological complaints were recruited as a control group.

Sample collection and bacterial isolation

Of all the subjects, the fresh stool samples were collected in a sterile plastic container and refrigerated at 4°C immediately until transferred to the laboratory within 24 hours. After transportation, all samples were homogenized and divided into two sterile tubes with 1.5 g of sample each. One portion of fecal samples was frozen at -80°C until DNA extraction and the other part were immediately cultured in man rogosa sharp (MRS) broth (Biolife, Milano, Italy) and incubated at 37°C for 48 hours under a microaerophilic condition (EZ-Campy System, Maryland, USA) with a gas mixture (5% O_2, 10% CO_2, and 85% N2). The samples were then s-cultured on MRS agar (Biolife, Milano, Italy) and incubated at 37°C for 48 hours for selective lactobacilli outgrowth under microaerophilic conditions. The bacterial colonies that developed on the plates were subjected to gram staining, morphology examination, and catalase activity, and only those that were catalase-negative and gram-positive rod-shaped morphology were considered as tentative the lactobacilli and selected for further studies.

Reference strains

The reference strains used to validate the study tests were composed of: *L. reuteri* ATCC 23272, *L. acidophilus* ATCC 4356, *L. plantarum* ATCC 8014, *L. rhamnosus* ATCC 7469, *L. paracasei* ATCC 25598, *L. casei* ATCC 39392, and *L. fermentum* ATCC 9338. All reference strains were grown in MRS broth (Biolife, Milano, Italy) for 24 hours at 37ºC under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N2).

DNA isolation

As previously described[9], bacterial DNA was extracted for species-specific PCR and RAPD tests, with some modifications: the bacterial isolates were freshly grown until an OD 600 nm about 1.0 in MRS broth, and one milliliters of each culture was centrifuged at 10,000 × g for 5 min. The bacterial pellets were washed twice with TE buffer (Tris-HCl [10 mM]: EDTA [1 mM]) and resuspended in 200 µl of TE buffer. The cells were then treated for 30 min at 37°C with lysozyme (2.5 mg/mL) (Qiagen, Hilden, Germany) and Proteinase K (250 mg/mL) (Qiagen, Hilden, Germany) to ensure complete degradation of bacterial cell walls. The suspension vortexed and subjected to a heating temperature of 100°C for 15 min using Incublock microtube incubators (Denville Scientific, USA). After boiling, the suspension was cooled immediately to -20°C for 15 minutes and then centrifuged for 5 minutes at 14,000 rpm. The supernatants were transferred to new microtubes and were used as DNA. The extracted DNA was then stored at -20 °C until analysis. Fecal DNA was extracted using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Genus-specific and species-specific qualitative PCR

The oligonucleotide primers specifications for lactobacilli detection are listed in TABLE 1[10-15]. Molecular identification of isolates at the genus level was carried out by qualitative PCR amplification of 16S-23S ribosomal RNA intergenic spacer region (ISR) according to the protocol of Turková[16]. After confirming the genus, species-specific qualitative PCR was employed for the identification of seven different *Lactobacillus* species. PCR reactions were performed in a total volume 25 µl, containing 12.5 µl of 1X PCR Master Mix (Ampliqon, Denmark), 3 µL of DNA template, 10 pmol of each primer, and distilled water to complete the PCR reaction volume. The thermal cycler program comprising an initial

TABLE 1. Primers of genus and species-specific PCR.

Specificity	Primers	Sequence (5'–3')	Annealing T (°C)	Amplicon length (bp)	Reference
Lactobacillus spp.	LbLMA 1rev R16-1	CTCAAAACTAAACAAAGTTTC CTTGTACACACCGCCCGTCA	55	250	[10]
L. casei	LcasF LcasR	TTTGAGGGGACGACCCTCAAGCA CGCCGACAAGCTATGAATTCACTTG	55	339	[11]
L. paracasei	LpacaF LpacaR	GATATTGCCTTCACGGTTTCAC TTGAARTCAACCATTGAGCG	65	125	[12]
L. rhamnosus	LrhamF LrhamR	GACTTCTCAACCAGCAGCGCAGA TGCATTTCCCCGCTTTCATGACT	68	801	[13]
L. fermentum	LFerF LFerR	GCCGCCTAAGGTGGGACAGAT CTGATCGTAGATCAGTCAAG	55	276	[14]
L. plantarum	Lpla-3 Lpla-4	ATTCATAGTCTAGTTGGAGGT CCTGAACTGAGAGAATTTGA	59	248	[15]
L. reuteri	Lreu-1 Lreu-4	GGCGGCTGTCTGGTCTGCAA GCTTGTTGGTTTGGGCTCTTC	55	303	[15]
L. acidophilus	LacidoF LacidoR	TGCAAAGTGGTAGCGTAAGC CCTTTCCCTCACGGTACTG	47	207	[15]

denaturation step at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, primer annealing (temperatures are given in TABLE 1) for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The amplification was performed in a C 1000 Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc.). All amplification products were separated by electrophoresis on 2% agarose gel, stained with 0.5 µg/mL ethidium bromide (Sinaclon, Tehran, Iran) and visualized under a UV transilluminator (ProteinSimple, San Jose, CA, USA). In every PCR run, a reagent blank, which contained all components of the reaction mixture with the exception of the DNA template, was included as the negative control.

Strain typing by RAPD-PCR analyses

The primer M13 (GAGGGTGGCGGTTCT)[17] was used for amplification. The PCR reaction was carried out in a total volume of 25 µL, containing 2 µL of primer M13 (10 pmol µL−1), 12.5 µl of 1X PCR Master Mix (Ampliqon, Denmark), 3 µL of genomic DNA (10 ng µL−1), 7.5 µL sterile deionized water. The amplification was carried out in the Eppendorf thermocycler 5530 (Roche, Mannheim, Germany) by using the following program: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, primer annealing for 1 min at 45°C, and primer extension for 1 min at 72°C with a final extension period at 72°C for 10 min. PCR products were then separated by electrophoresis on 2% (w/v) agarose gel using 1x TBE buffer containing 0.5 µg /mL ethidium bromide (Sinaclon, Tehran, Iran) at 80 V for 2 h. A DNA marker 100bp ladder (Ampliqon, Denmark) was used as a molecular weight standard. RAPD-PCR fingerprints analysis and clustering were processed by the BioNumerics software version 6.6 (Applied Maths, Belgium). Similarities were calculated using the Pearson correlation coefficients. The dendrograms were acquired by means of the clustering algorithm of the Unweighted Pair Group Method using Arithmetic Average (UPGMA).

Sequencing and analysis of PCR products

To confirm the identification of the *Lactobacillus* isolates that investigated by using qualitative PCR, as well as to verification of strain specificity of the primers, purified amplicons were sequenced using the species-specific primers. From each cluster obtained by the RAPD method, one to five isolates were selected for sequencing. This analysis was conducted at the Genetics research laboratory of Rajaie Cardiovascular, Medical and Research Center, Tehran, using their standard protocol. All the obtained sequences were verified in the GenBank DNA database by using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (NCBI).

Quantitative real-time PCR

To measure the quantity of total lactobacilli in each fecal sample, qPCR was performed using genus-specific primer sets (TABLE 1). Amplifications were conducted in an ABI StepOne (Applied Biosystems, USA) according to the previously described protocol[18]. Standard curves were generated using serial 10-fold dilutions (10^1 to 10^7 copies) of fecal bacterial DNA of known concentration. The lactobacilli copy numbers in fecal samples were determined from the values of the threshold cycle (Ct) and represented as the amount of bacteria/gram of feces.

Statistics

The qualitative variables were analyzed using the chi-square test and the exact Fisher test. The significance level was considered less than or equal to the test-wise error rate of 0.05 in all tests. All statistical test was done using SPSS software (version 24; SPSS, Inc., Chicago, IL, USA), version 2016 of Microsoft Office Excel, and version 8 of GraphPad Prism (GraphPad Software, Inc.).

Ethical consideration

The Institutional Review Board (IRB) and Research Ethics Committee of Medical Faculty of the University approved this project (Code: IR.AJUMS.REC.1397.433) and the necessary permission for collecting the sample was granted.

RESULTS

Lactobacillus profiling by qualitative PCR

In the present study, out of 80 stool samples, culture-positive 65 (81.3%) ones were analyzed by genus and species-specific qualitative PCR from which 25 (38.46%) and 40 (61.54%) samples were collected from constipated and non-constipated children, respectively. In total seven different *Lactobacillus* species were identified in both healthy and patient groups. The most prevalent species were *L. paracasei* 21 (32.3%) followed by *L. plantarum* 18 (27.7%) and the less prevalent species was *L. acidophilus* 3 (4.6%). There were no statistically significant differences in the prevalence of Lactobacillus species between constipated and non-constipated children (TABLE 2). Each of the twenty-five of constipated patients was colonized by only one *Lactobacillus* strain, whereas five out of all healthy subjects were colonized by two distinct genetic strains in the stool specimen.

TABLE 2. Prevalence of *Lactobacillus* species, detected by Genus and species-specific PCRs.

Non constipated N (%)		Constipated N (%)	Total N (%)	*P*-value
Lactobacillus spp.	40 (61.5)	25 (38.5)	65 (81.3)	0.264*
L. plantarum	10 (15.4)	8 (12.3)	18 (27.7)	0.637*
L. paracasei	13 (20)	8 (12.3)	21 (32.3)	0.275*
L.rhamnosus	6 (9.2)	3 (4.6)	9 (13.8)	0.508€
L. reuteri	3 (3.1)	2 (4.6)	5 (7.7)	>0.999€
L. casei	2 (3.1)	2 (3.1)	4 (6.2)	>0.999€
L. fermentum	3 (4.6)	2 (3.1)	5 (7.7)	>0.999€
L. acidophilus	3 (4.6)	0	3 (4.6)	>0.999€

*Pearson's chi-square test; €: Fisher's exact test.

RAPD-PCR analysis

After initial identification of *Lactobacillus* using species-specific PCR method, the genetic heterogeneity of strains belonging to each of seven distinct species was determined by the numerical evaluation of DNA profiles obtained with RAPD–PCR technique in both groups of patients and healthy subjects. Cluster analysis was performed similarly with UPGMA estimates, and the determined dendrograms obtained revealed the genetic relationships between lactobacilli isolates and reference strains. In total, seven main clusters were determined at an arbitrarily chosen similarity level of 45% or above (FIGURE 1) and were allocated to each of the seven intended species in the study. The clusters of *L. paracasei* and *L. plantarum* species composed of three and four sub-cluster, respectively, whereas clusters of *L. fermentum*, *L. rhamnosus*, *L. casei*, *L. reuteri*, and *L. acidophilus* species have only two sub-

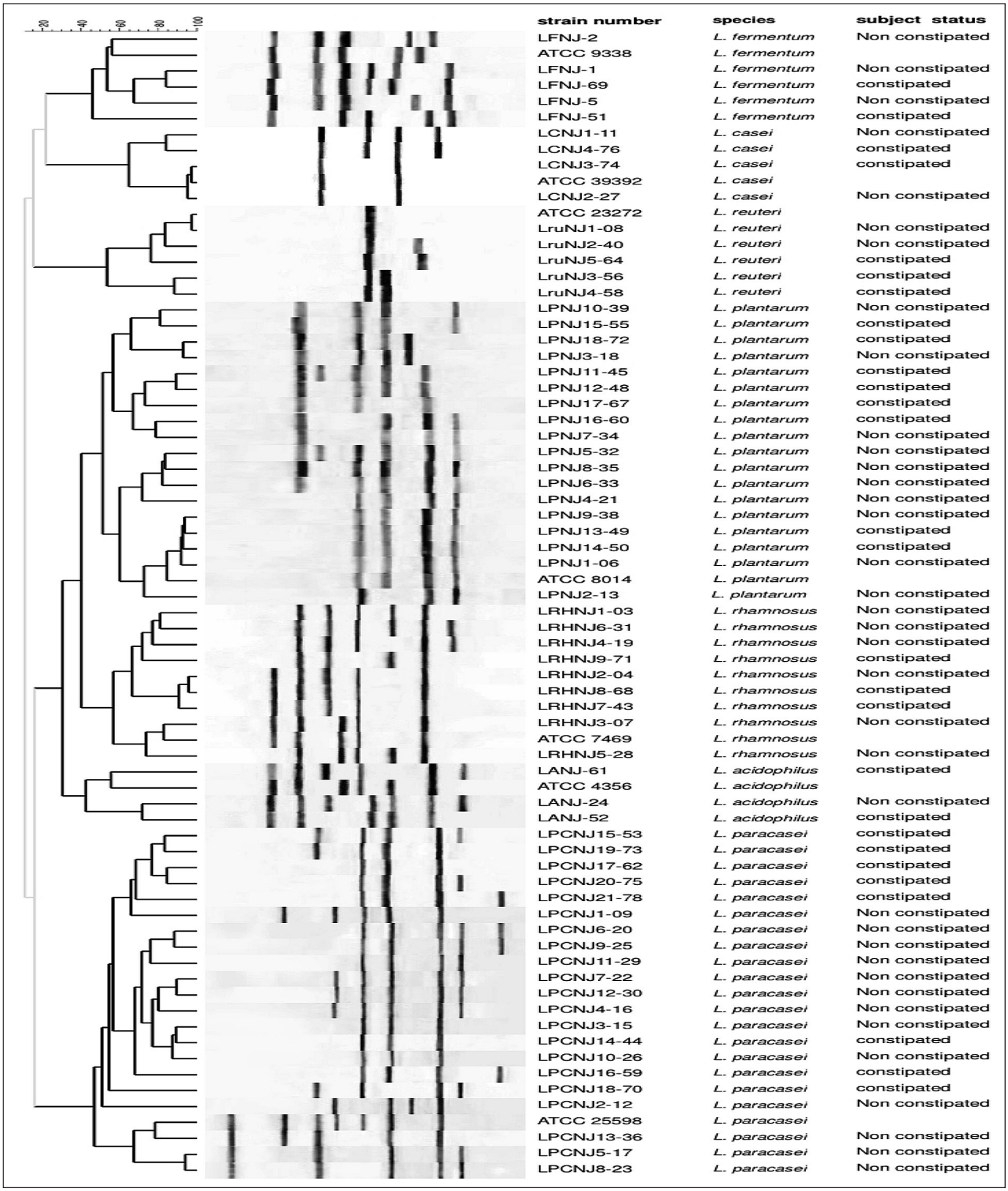

FIGURE. 1. Dendrogram generated from RAPD-PCR patterns using the M13 primer from clinical and reference *Lactobacillus* species. Strains belonging to each of seven *Lactobacillus* species formed single specific clusters at similarity levels exceeding 60%.

clusters, each of them has an internal correlation coefficient of about 60% to 98%. As shown in FIGURE 1, strains isolated from constipated patients with strains obtained from the control group were genotypically closely related and have a similarity coefficient of more than 90%.

DNA sequencing and accession numbers

Sequencing of the target gene of selected *Lactobacillus* isolates of each species was done to confirm the species as indicated by species-specific PCR. Totally 22 isolates were sequenced and submitted to the GenBank database under the accession numbers MN190691 – MN190699, MN184661 – MN184668 and MN186307 – MN186311.

Quantification of lactobacilli in feces

Using genus-specific qPCR assay, the amount of *Lactobacillus* spp. in the feces of both constipated and healthy children was further assessed. The results of genus-specific quantitative PCR showed that in healthy controls, the total counts of lactobacilli were significantly higher in comparison with constipated children (mean 6.26 (SD 0.3) $\log_{10}$ CFU/gram of feces versus mean 4.33 (SD 0.63) $\log_{10}$ CFU/gram of feces; P<0·0001) (FIGURE 2).

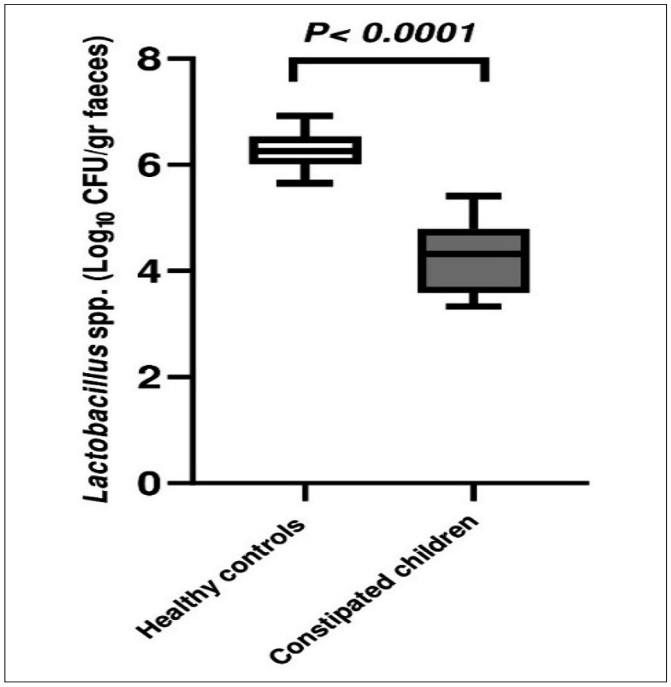

FIGURE 2. Box and whiskers plots presenting the amounts of *Lactobacillus* spp. in constipated children and healthy controls by real-time PCR. The results of Mann Whitney test showed that there was a significant difference between log10 CFU/gram feces in both groups.

DISCUSSION

The potential association between functional gastrointestinal disorder and probiotics is attractive and opens interesting therapeutic and preventive strategies in the probiotic and gastrointestinal researches[6]. Improved gastrointestinal motility with some type of *Lactobacillus* (especially *L. reuteri* and *L. acidophilus*) and *B. bifidum* were observed in some experiments[19-22]. In the present study, based on species-specific PCR analysis, no significant differences were observed in the prevalence of intestinal *Lactobacillus* species in healthy and constipated children. Moreover, in agreement with previous results[23,24], we found that in the healthy subjects and constipated group, *L. paracasei* and *L. planetarium* species were predominant types of lactobacilli (prevalence of these species, 32.3% and 27.7%, respectively).

Several systematic reviews and meta-analyisis do not recommend routine use of probiotics in the treatment of constipation[25-28].

Even though many useful genotyping techniques have been applied for differentiating and genetic diversity of lactobacilli strains, these methods have pitfalls and limitations such as cost, complexity, and time-consuming[29]. RAPD PCR, by contrast, is rapid, simple, and cost-effective and has been found useful for differentiation between species of *Lactobacillus*. In this study, RAPD-PCR with M13 primer was employed for differentiation between strains and assessment of intraspecies variability. Analysis of the consensus dendrograms (FIGURE 1) displayed that very closely genetically related strains of the same species, presumably belonging to the same clonal generation, which sorted at a similarity coefficient >90%. This was similar to the earlier findings reported by other authors[30,31].

Until now, the 16S rRNA gene sequencing has been widely used as a valuable tool for identifying bacteria at the species level due to its universality and technical easiness[32]. In a recent study, 98.65% was proposed by Kim et al. as the species demarcation cut-off value for 16S rRNA gene sequence similarity[8]. On the other hand, since the similarity of 16S rRNA gene sequences between closely related species, such as different *Lactobacillus* species can reach more than 99%, may not be the suitable target to design a very specific primer sets for this gene[33]. To resolve this problem, protein-encoding genes, as well as intergenic sequences of the 16S–23S rRNA spacer region, have been proposed as two alternatives to 16S rRNA gene sequence[12,34]. In this study, it has been shown that 16S–23S rRNA and protein-encoding genes are suitable targets for the specific quantification of *Lactobacillus* species. It was also found that all the seven primer pairs used in our study are capable to identify corresponding genes from closely related *Lactobacillus* species in the fecal sample.

In recent years, many researchers have been using the SYBR green qPCR approach to identify various non-predominant organisms in stool specimens[18,35-37]. In this research, the fecal lactobacilli flora colonizing the intestines of both healthy and patient groups was examined using genus-specific qPCR primers. In the present study, lactobacilli were detected by the culture and conventional PCR methods in 65 (81%) of the fecal samples, and by quantitative PCR in all samples. The present results confirm the previous reports[38,39], which indicates the qPCR technique's higher diagnostic capability than the other two approaches in complex samples. Following our study, Štšepetova et al.[39] used the real-time PCR approach for quantification of total lactobacilli, as well as conventional PCR for assessing intended *Lactobacillus* species in the human feces. Furthermore, they also showed that there was no correlation distinguished between the type and number of species and the count of lactobacilli. Overall, based on the qPCR results, we observed a significantly lower quantity of lactobacilli in patients with functional constipation compared to healthy controls, which was in agreement with previous studies that showed low quantities of lactobacilli in constipated children using culture methods[40] and SYBR green qPCR[41].

CONCLUSION

Finally, we found that children suffering from functional constipation had a smaller quantity of total lactobacilli per milligram of stool compared to healthy controls. It can be concluded from our findings that the mere existence of lactobacilli in the colon is not sufficient to impact and prevent certain gastrointestinal disorders such as childhood functional constipation, and their amounts plays a more pivotal role. Our future studies will focus on quantifying the *Lactobacillus* species in the gut of healthy subjects compared with constipated children by qPCR assay, as well as on elucidating their probiotic properties to prevent functional constipation.

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Authors' contribution

Jomehzadeh N: performed the laboratory work; wrote the article and revising it critically for important intellectual content. Javaherizadeh H: performed the eligibility screening. Amin M: conceived and designed the study. Rashno M: analysed data and co-edited paper. All authors have read the contents of the manuscript and confirmed the aforementioned subject.

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RESUMO – Contexto – A investigação das cepas bacterianas específicas do intestino, incluindo lactobacilos, é essencial para a compreensão da etiologia bacteriana da prisão de ventre. **Objetivo –** Este estudo teve como objetivo comparar a prevalência e a quantidade de lactobacilos intestinais em crianças constipadas e controles saudáveis. **Métodos –** Foram recrutadas quarenta crianças que preenchem os critérios de Roma IV para prisão de ventre funcional e 40 controles saudáveis. As amostras fecais foram analisadas utilizando-se uma reação da cadeia de polimerase específica da espécie, seguida por DNA polimórfico amplificado aleatório e PCR quantitativo em tempo real. **Resultados –** Foram detectadas sete espécies diferentes de lactobacilos. Dos 80 voluntários, 65 (81,3%) eram cultura em PCR específico de espécies, dos quais 25 (38,46%) crianças constipadas e 40 (61,54%) indivíduos saudáveis. As espécies mais prevalentes foram *L. paracasei* 21 (32,3%) seguidas por *L. plantarum* 18 (27,7%) entre grupos saudáveis e de pacientes. A análise dos dendrogramas do RAPD mostrou que cepas isoladas de crianças constipadas e não constipadas têm coeficientes de similaridade superiores a 90%. Os ensaios qPCR demonstraram que as crianças constipadas apresentavam uma quantidade menor de população total de lactobacilos (por grama de fezes) do que os controles saudáveis. **Conclusão –** Nossos achados mostraram que a mera existência de várias espécies de *Lactobacillus* no intestino não é suficiente para prevenir alguns distúrbios gastrointestinais, como a prisão de ventre funcional, e sua quantidade desempenha um papel mais importante.

Palavras-chave – Prisão de ventre; fezes; *Lactobacillus*; prevalência.

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