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Modifications of mice gut microflora following oral consumption of Lactobacillus acidophilus and *Bifidobacterium bifidum* probiotics

Hossein KHAVARI-DANESHVAR[1], **Maryam MOSAVI**[1], **Hamid KHODAYARI**[1], **Ebrahim RAHIMI**[2], **Peyman RANJI**[1], **Amir Hossein MOHSENI**[3], **Reyhaneh MAHMUDIAN**[4], **Farzad SHIDFAR**[4], **Shahram AGAH**[4,*], **Ali Mohammad ALIZADEH**[1,*,**]
[1]Cancer Research Center, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Food Hygiene, College of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
[3]Research and Development Department, Zist Takhmir Company, Tehran, Iran
[4]Colorectal Research Center, Iran University of Medical Sciences, Tehran, Iran

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Background/aim: Thirty male BALB/c mice were equally divided into three groups: control, *L. acidophilus*, and *B. bifidum* for the assessment of the probiotics' stability in the gut microflora.

Materials and methods: First, the gut microflora of the mice was checked every 3 days (days 3, 6, 9, and 12) without probiotic consumption, and then the mice were daily given orally 1.5 g of probiotics in 30 cc of drinking water. The consumption of probiotics was then stopped for recovery and then the consumption continued for 5 months.

Results: On day 9 after the consumption of the probiotics, *L. acidophilus* and *B. bifidum* were significantly increased from 4% to 83% and from 1% to 61%, respectively. *L. acidophilus* count showed no significant decrease at the end of 5 months compared to day 9 of probiotic consumption (74%), but *B. bifidum* count was dramatically decreased to 45% and 36% at the end of 1 and 5 months, respectively.

Conclusion: Our results revealed that, unlike *B. bifidum*, the amount of *L. acidophilus* remained almost unchanged in the long term, indicating more stability of *L. acidophilus* than *B. bifidum* in the gut microflora.

Key words: *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, probiotic, gut microflora

1. Introduction

The human gastrointestinal (GI) tract is covered with more than 500 different species of bacteria that can influence the host's pathophysiology. Probiotics are the beneficial nonpathogenic bacteria used as biotherapeutic agents for the prevention or treatment of some diseases (1,2). In this regard, the essential point is to demonstrate a distinct health advantage attained by consumption of a specific probiotic strain that cannot be compared even to other strains of the same species (3). Herein, overgrowth of pathogenic organisms within the GI tract, stimulation of intestinal immunity, and production of essential nutrients and/or bioactive food components might be related to the risk of developing neoplastic diseases such as cancer (4–6). Thus, gut microflora may influence the multiple processes associated with a change in cancer risk, and consequently removal of the inflammatory bacteria by probiotic agents is a potential mechanism to modulate disease severity (3,7).

A number of in vitro and in vivo studies have demonstrated that the consumption of specific probiotics such as *bifidobacterium* and *lactobacillus* can modulate the intestinal bacteria (8). Undeniably, greater attention to the duration of beneficial probiotic stability is required for health promotion (4,9). It seems that based on the short- and long-term utilization of various probiotics for gut microflora modification, they can be used in different approaches regarding various diseases (6). The specific probiotic strains with short-term storage can be used for treatment of some disorders such as diarrhea (8,10). The critical point is to demonstrate the long-term prevention and treatment of some diseases like cancer that can be achieved by using specific probiotic strains that can be replaced and stored long term in the gut (11).

* These authors contributed equally to this work
** Correspondence: aalizadeh@sina.tums.ac.ir

The aim of the present study was to evaluate the effects of *Bifidobacterium bifidum* (*B. bifidum*) and *Lactobacillus acidophilus* (*L. acidophilus*) probiotics on concentration of mice gut microflora. Increasing of *B. bifidum* and *L. acidophilus* through specific dietary intervention of Bla/016P/M and Lac/002P/M probiotics may cause inhibition of initiation and development of GI disorders. Moreover, the existence and stability of concentrations of *B. bifidum* and *L. acidophilus* in the gut microflora should be evaluated during several months of probiotic consumption.

2. Materials and methods

2.1. Materials

B. bifidum probiotic (Bla/016P/M), from a traditional product (yogurt), and *L. acidophilus* probiotic (Lac/002P/M), from CHR Hansen (La5), were gifted by Zist Takhmir Supplements Company (Tehran, Iran). BHI broth (brain heart infusion broth), NAT agar (nalidixic acid tween agar), and EMB agar (eosin methylene blue agar) were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Preparation of media and method of culture

First 3.7 g of BHI broth and 10.5 g of EMB agar powder were separately dissolved in 100 and 300 cc of water, respectively, according to the manufacturers' instructions. Next, 43 g of NAT agar and 42 g of BHI agar powder were separately dissolved in 800 cc of water. Then 1 cc of the resulting solution was taken and the autoclave processing was done at 121 °C and 15 atmosphere pressure for 15 min. The anaerobic bacteria culture in the fecal sample was done on NAT agar and BHI agar, and then incubated in an anaerobic jar using a gas pack system at 37 °C. The aerobic bacteria culture was performed on EMB agar and BHI agar, and followed by incubation at 37 °C with the same dilution.

2.3. Staining assay

Before starting the Gram staining, two slides were prepared from each culture plate of bacteria. Then a crystal violet stain was applied to the heat-fixed smear of the bacterial culture. Heat fixation is mostly used to affix the bacteria to the slide so that they do not rinse out during the staining procedure. After that, the sufficient iodine solution was added in order to bind to crystal violet and trap it in the cell. The next step was rapid decolorization with ethanol/acetone. Finally, Safranin was used as a counterstain.

2.4. Animal study

Male inbred BALB/c mice (6–8 weeks old, purchased from Iran Pasteur Institute) were maintained under 12-h dark and light cycles, and were given access to food and water ad libitum. The procedures were done in accordance with the guidelines for the care and use of laboratory animals of Tehran University of Medical Sciences.

2.5. Study design

Thirty mice were equally divided into control, Lac/002P/M, and Bla/016P/M groups for assessment of the probiotics' stability in the long term. In this context, the gut microflora was evaluated every 3 days (days 3, 6, 9, and 12), and then the mice were daily given orally 1.5 g of probiotics (1×10^9 cfu/g Lac/002P/M and 1×10^9 cfu/g Bla/016P/M) in 30 cc of drinking water over 12 days for gut microflora replacement with the probiotics. The probiotics consumption was stopped for the same duration to achieve gut microflora recovery; then the probiotic feeding continued for 5 months (6).

2.6. Fecal sampling

Fecal samples of mice were collected before and after treatment in separate sterile tubes including 1 cc of BHI broth. Different dilutions (1:10, 1:100, and 1:1000) of each sample were transferred onto a BHI agar plate and cultured with the standard pour-plate method. After incubation and comparison of the colony count results, the dilution 1:1000 was selected as the optimum. The optimum specimens were placed on the selective culture plates and incubated anaerobically using a gas pack system at 37 °C for 72 h and aerobically at 37 °C for 24 h with the streak-plate method. All the bacterial isolates were identified by morphological study, Gram staining, and different biochemical tests such as catalase, oxidase, and carbohydrate fermentation tests (12–16). Moreover, a combination of RT-PCR and denaturing gradient gel electrophoresis was used for the validation of *L. acidophilus* existence in the gut (17,18). Table 1 shows the sequences of the specific primers used for this method.

Table 1. The specific primer sequences used for identification of *Lactobacillus acidophilus* with RT-PCR method.

Lactobacillus acidophilus	Sequences
P1B16	AGA GTT TGA TCC TGG CTC AG
MLB16	GGC TGC TGG CAC GTA GTT AG
Ss2	CACGGATCCTACGGGTACCTTGTTACGACTT
HE1	AGCAGATCGCATGATCAGCT

2.7. Statistical analysis

Depending on the number of groups to be compared within each trial and depending on the P-value of the Kolmogorov–Smirnov test of normality, a t-test, one-way ANOVA, or a nonparametric test was used for data analyses. Differences between groups in the bacteria amounts were estimated by analyzing the area under the curve. A level of P < 0.05 was considered to be statistically significant. Statistical analysis was done using SPSS (version 13.0; SPSS Inc., Chicago, IL, USA).

3. Results

The specific media were used for the aerobic and anaerobic samples during the pour-plate and the streak-plate techniques to determine the normal microflora count in each sample. Identification of normal microflora was performed by morphological study, Gram staining, and different biochemical tests. The microflora assay on normal animals before probiotic consumption was done every 3 days (days 3, 6, 9, and 12) (Tables 2 and 3). In general, it is notable that 99.6% of microflora were anaerobic bacteria (of these, the maximum percentage of 52% belonged to *Bacteroides* spp.) and 0.4% were aerobic bacteria (68% of these cases belonged to *E. coli*).

Microflora concentration in the group consuming Lac/002P/M probiotics consisted of 99.6% anaerobic bacteria (with the maximum percentages of 83% and 10.5% for *L. acidophilus* and *Bacteroides* spp., respectively) and 0.4% aerobic bacteria on day 9 of treatment (Table 2). *L. acidophilus* existence in the gut was verified using a combination of RT-PCR and DGGE (Figure). Moreover, 99.6% anaerobic bacteria (the maximum rates belonged to *B. bifidum* with 61% and *Bacteroides* spp. with 27%) and 0.4% aerobic bacteria in microflora concentrations were detected in the group consuming Bla/016P/M on day 9 of treatment (Table 3). The mean values of *B. bifidum* and *L. acidophilus* populations were significantly increased from 1% and 4% to 61% and 83%, respectively (P < 0.05) (Tables 2 and 3). Moreover, the consumption of the probiotics was stopped for 12 days to achieve gut microflora recovery. Our data showed that on day 6 after pausing of the probiotic feeding, the normal gut microflora returned to its normal count (the same as before probiotic consumption). The consumption of the probiotics was continued for 5 months and microflora assays were performed monthly. At the end of 1 month of consumption, *Lactobacillus acidophilus* remained unchanged (83%) (Table 2), but *Bifidobacterium bifidum* was decreased from 61% to 45% (P < 0.05) (Table 3). At the end of 5 months, *Lactobacillus acidophilus* was not significantly declined compared to day 9 of probiotic consumption (83% to 74%) (P > 0.05) (Table 2), but *Bifidobacterium bifidum* was significantly decreased from 61% to 36% (P < 0.05) (Table 3).

4. Discussion

The purpose of the present study was to determine whether persistent consumption of probiotics can modify the gut microflora in the long term. The results demonstrated that treatment with Lac/002P/M and Bla/016P/M probiotics could significantly increase *L.*

Table 2. Mice microflora concentration before and after Lac/002P/M consumption.

Type of bacteria	Total (%)	Bacteria spp.	Before treatment (%)	Day 9 of treatment (%)	After 1 month of treatment (%)	After 5 months of treatment (%)
Aerobic	≈ 0.4	*E.coli*	68	68	68	68
		Other coliforms	32	32	32	32
Anaerobic	≈ 99.6	*Bacteroides* spp.	52	10.5	10.5	15.5
		Prevotella spp.	14	2.5	2.5	4.5
		Clostridium spp.	17	2	2	3
		Peptostreptococcus spp.	11	1	1	2
		Lactobacillus acidophilus	4	83 *	83 *	74 *
		Bifidobacterium bifidum	1			
		Other bacteria	1	1	1	1

Gut microflora was assessed every 3 days (days 3, 6, 9, and 12) after oral probiotic consumption, and then continued for 5 months. On day 9 of probiotic consumption, *Lactobacillus acidophilus* was significantly increased from 4% to 83% (P < 0.05), but its count showed no significant change at the end of 5 months compared to day 9 of probiotic consumption (74%) (P > 0.05). *P < 0.05 compared to before treatment.

Table 3. Mice microflora concentration before and after Bla/016P/M consumption.

Type of bacteria	Total (%)	Bacteria spp.	Before treatment (%)	Day 9 of treatment (%)	After 1 month of treatment (%)	After 5 months of treatment (%)
Aerobic	≈ 0.4	*E.coli*	68	68	68	68
		Other coliforms	32	32	32	32
Anaerobic	≈ 99.6	*Bacteroides* spp.	52	27	38	42
		Prevotella spp.	14	4	9	11
		Clostridium spp.	17	4	4	5
		Peptostreptococcus spp.	11	3	3	5
		Lactobacillus acidophilus	4			
		Bifidobacterium bifidum	1	61*	45 *,#	36 *,#
		Other bacteria	1	1	1	1

Gut microflora was assessed every 3 days (days 3, 6, 9, and 12) after oral probiotic consumption, and then continued for 5 months. *Bifidobacterium bifidum* was significantly increased from 1% to 61% onday 9 after probiotic consumption ($P < 0.05$), but was dramatically decreased to 45% and 36% at the end of 1 and 5 months, respectively ($P < 0.05$). *$P < 0.05$ compared to before treatment.
#$P < 0.05$ compared to day 9 of probiotic consumption.

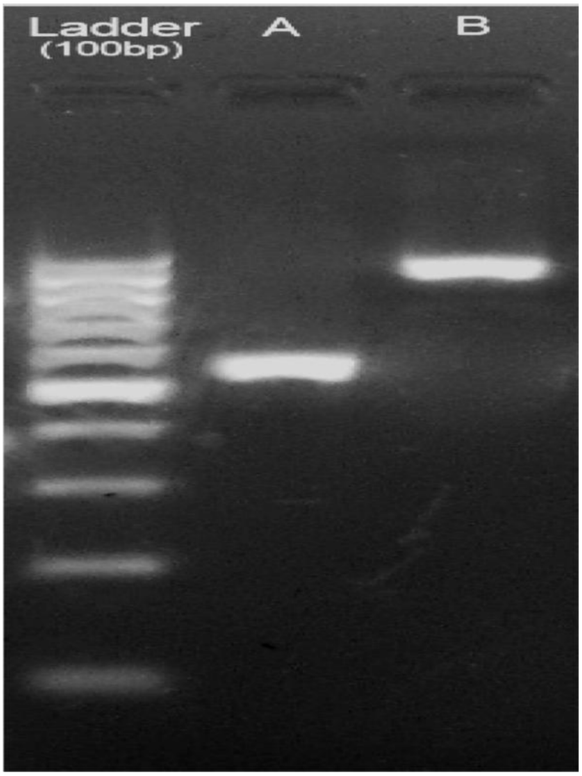

Figure. *L. acidophilus* existence in the gut was verified using a combination of RT-PCR and denaturing gradient gel electrophoresis.

acidophilus and *B. bifidum* concentrations from 4% and 1% to 83% and 61%, respectively, in the gut of mice on day 9 of treatment. Existence and remaining of probiotics were also observed in microflora concentration 1 and 5 months after consumption of probiotics (83% and 74% for *L. acidophilus* and 45% and 36% for *B. bifidum*, respectively). Our results revealed that, unlike *B. bifidum*, *L. acidophilus* amount remained almost unchanged during the long-term probiotic consumption. Thus, our data may indicate more stability of *L. acidophilus* than *B. bifidum* in the gut microflora in the long term.

The collected data from previous studies demonstrated that alterations in the gut microflora can lead to GI disorders (19). Dietary components may influence microflora balance and modification (4,20), which can in turn reduce the incidence of several problems in the GI (3). Undeniably, greater attention is needed about the exposure duration of beneficial probiotics for health promotion (4,9). It seems that based on the ability of various probiotics in the gut microflora modification in the long or short term, they can have different effects on various diseases. In this regard, several (pre) clinical studies have demonstrated that the application of probiotics can have beneficial effects on diarrhea, intestinal infections, inflammatory bowel disease, and irritable bowel syndrome (8,21). Studies have shown that using specific probiotic strains such as *B. bifidum* can probably be useful for the treatment of gut-related diseases in the short term (8,10).

In addition, well-controlled clinical studies have revealed that *L. rhamnosus* GG and *B. animalis* Bb12 probiotics can reduce the duration of acute rotavirus diarrhea (22,23).

On the other hand, the critical point is to demonstrate the best and long-term prevention and treatment of diseases like cancer and polyps (6), which may be achieved using specific probiotic strains such as *L. acidophilus* that can be replaced and stored in the long term in the gut (11). In our study, a high amount of *L. acidophilus* was observed in the gut microflora at the end of 5 months after probiotic consumption. In this regard, the results of another study indicated that the long-term presence and remaining of *L. acidophilus* can have a critical role in modulating colorectal cancers (3). An in vivo study demonstrated that *L. acidophilus* consumption decreased the ratio of aberrant crypt foci in rats with a high-fat diet containing the carcinogen (24). Furthermore, a reduction in tumor progression in the small intestine was also observed in mice receiving the probiotic yogurt formulation containing microencapsulated live *L. acidophilus* cells (25). In addition, the gavage of *L. acidophilus* probiotics inhibited tumor growth in an orthotopic mouse model of colon cancer (26). The collected data from the mentioned studies suggest that the storage of *L. acidophilus* in the gut in the long term may be essential for prevention and/or treatment of gut-related disease including colon cancer.

Finally, the present study demonstrated a greater stability in *L. acidophilus* than *B. bifidum* in the gut microflora of mice in the long term (5 months) consumption of probiotics. It seems that based on the ability of various probiotics to modify the gut microflora in the long or short term, they can have some beneficial effects on various gut-related diseases.

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