ABSTRACT

We conducted a randomized double-blind, placebo-controlled multicentric study to investigate the influence of a synbiotic fermented milk on the fecal microbiota composition of 30 adults with irritable bowel syndrome (IBS). The synbiotic product contained *Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* ssp. *lactis* BB-12, *Streptococcus thermophilus*, and dietary fiber (90% inulin, 10% oligofructose), and a heat-treated fermented milk without probiotic bacteria or dietary fiber served as placebo. Stool samples were collected after a run-in period, a 4-wk consumption period, and a 1-wk follow-up period, and were subjected to real-time PCR and 16S rDNA profiling by next-generation sequencing. After 4 wk of synbiotic (11 subjects) or placebo (19 subjects) consumption, a greater increase in DNA specific for *L. acidophilus* La-5 and *B. animalis* ssp. *lactis* was detected in the feces of the synbiotic group compared with the placebo group by quantitative real-time PCR. After 1 wk of follow-up, the content of *L. acidophilus* La-5 and *B. animalis* ssp. *lactis* decreased to levels close to initial levels. No significant changes with time or differences between the groups were observed for *Lactobacillus*, *Enterobacteriaceae*, *Bifidobacterium*, or all bacteria. The presence of viable BB-12- and La-5-like bacteria in the feces resulting from the intake of synbiotic product was confirmed by random amplification of polymorphic DNA (RAPD)-PCR. At the end of consumption period, the feces of all subjects assigned to the synbiotic group contained viable bacteria with a BB-12-like RAPD profile, and after 1 wk of follow-up, BB-12-like bacteria remained in the feces of 87.5% of these subjects. The presence of La-5-like colonies was observed less frequently (37.5% and 25% of subjects, respectively). Next-generation sequencing of 16S rDNA amplicons revealed that only the percentage of sequences assigned to *Strep. thermophilus* was temporarily increased in both groups, whereas the global profile of the fecal microbiota of patients was not altered by consumption of the synbiotic or placebo. In conclusion, daily consumption of a synbiotic fermented milk had a short-term effect on the amount and proportion of La-5-like strains and *B. animalis* ssp. *lactis* in the fecal microbiome of IBS patients. Furthermore, both synbiotic and placebo products caused a temporary increase in fecal *Strep. thermophilus*. 

Key words: synbiotic fermented milk, irritable bowel syndrome, metataxonomics, real-time PCR

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional disorder of the gastrointestinal tract (GIT) affecting 1 to 32% of adults worldwide (Canavan et al., 2014). In accordance with the Rome III criteria (Drossman, 2006), IBS is characterized by abdominal pain or discomfort and altered bowel function, and it can be further categorized into constipation-predominant, diarrhea-predominant, or mixed type (Longstreth et al., 2006). The disorder has a significant negative effect on the well-being and health of affected subjects, leading to considerable socioeconomic consequences. Recent therapeutic approaches in IBS include, in addition to various pharmaceutical agents and dietary...
Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (FAO-WHO, 2001; Hill et al., 2014). The most common source of probiotics is human indigenous microbiota. The interest in probiotics as possible therapeutics for IBS treatment arose from the observation that the fecal microbiota of IBS patients differs significantly from that of healthy subjects (Malinen et al., 2010; Quigley, 2011; Rajilić-Stojanović et al., 2011). Because studies suggest that dysbiosis (i.e., alteration of intestinal microbiota) plays a role in several diseases, including IBS, Benno et al. (2015) suggested the introduction of the term “dysbiotic bowel syndrome” to describe the effects and consequences of dysbiosis leading to symptoms associated with IBS. The potential of probiotics in the treatment of IBS is attributed to the ability of certain strains to regulate—either directly or indirectly through modulation of the host’s microbiota metabolism—the passage of food through the gut, visceral hypersensitivity, gas quantity in the gut, and immune responses. Amelioration of the symptoms by probiotic products has already been observed in several human clinical studies on IBS patients (Roberts et al., 2013; Konig and Brummer, 2014; Yoon et al., 2014).

A dietary prebiotic is “a selectively fermented, non-digestible food ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon host health” (Gibson et al., 2010). Probiotic bacteria are often combined with prebiotics in so-called synbiotic products. In accordance with the synbiotic concept, the prebiotics in such products may improve the survival and persistence of consumed probiotics in the gastrointestinal tract, by selectively stimulating their growth or by activating their metabolism (Gibson and Roberfroid, 1995; Gibson et al., 2010). Different synbiotic products are available on the market, such as lyophilized powders, nonfermented foods, or fermented dairy products. Fermented milk matrix offers several advantages compared with other products, such as better protection for ingested bacteria under gastrointestinal tract conditions (Saxelin et al., 2010).

Ingested probiotics form part of the “transient microbiota” that is present in our body for a relatively short time. However, although their permanent colonization is largely prevented by the colonization resistance of the resident microbiota, they may still affect the microbiome composition of the gut and may change the metabolic capacity of the resident gut communities (Derrien and Vlieg, 2015). The composition of the transient microbe is continuously changing under the influence of the gut environment, diet, and other factors associated with the host. It is therefore not surprising that changes in the microbiome after consumption of probiotics are not always observed, despite the conferred positive effects on health. Even though the mechanisms of the interaction between probiotics and the host have been studied in healthy and diseased people, they are still not completely understood. Previous studies showed changes in human gut microbiota after the ingestion of conventional yogurt (Garcia-Albiach et al., 2008), probiotic fermented milk (Savard et al., 2011; Filteau et al., 2013; Veiga et al., 2014), or other types of probiotic products (Ng et al., 2013; van Zanten et al., 2014; Yoon et al., 2014). There are a few reports on the effects of synbiotics including fermented milk-based and pharmaceutical formulations on the IBS patients (Tsuchiya et al., 2004; Andriulli et al., 2008; Cappello et al., 2013), but none of these studies was reported to be randomized double-blind, placebo-controlled, and multicentric.

Previous clinical trials with fermented milk products containing Lactobacillus acidophilus La-5, Bifidobacterium animalis ssp. lactis BB-12, or both, were carried out on healthy people only (Savard et al., 2011; Palaria et al., 2012; Filteau et al., 2013). Furthermore, the gut microbiota was examined in these studies only partially, mostly by plate counting, real-time quantitative (q)-PCR, terminal-RFLP analysis, or random amplification of polymorphic DNA (RAPD)-PCR. Today, however, more powerful methods based on next-generation sequencing (NGS) approaches are available to study the effect of ingested bacteria on the human gut microbiota. These approaches, including 16S rDNA phylogenetic microarray, 16S rRNA sequencing, shotgun metagenomics sequencing, or quantitative metagenomics, enable a more comprehensive insight into the entire microbiome. They have been successfully applied in some recent studies on IBS patients who consumed probiotic products containing a wide range of bacteria other than La-5 and BB-12 strains (Kajander et al., 2008; Ng et al., 2013; Veiga et al., 2014), and in a study that examined a multistrain probiotic milk-based drink also containing BB-12 (Kajander et al., 2008). Because molecular approaches based on analyses of total bacterial DNA do not provide any data about the viability or physiological state of the probiotic bacteria in the samples (essential in validating the efficacy of probiotics in the host), we combined high-throughput pyrosequencing and qPCR analyses based on bacterial 16S ribosomal gene sequences with RAPD-PCR analysis of the cultivable lactobacilli and bifidobacteria from feces in the present study.
The objective of this study was to investigate, by using qPCR and metataxonomics, the influence of a synbiotic fermented milk product containing *Lactobacillus acidophilus*, *Bifidobacterium animalis* ssp. *lactis*, and dietary fiber on the fecal microbiota composition of adults with IBS. Due to the lack of properly controlled clinical studies with fermented probiotic or synbiotic products, the results of this randomized double-blind, placebo-controlled multicentric study present a valuable contribution to the research of interaction between probiotics and the host.

**MATERIALS AND METHODS**

**Participants and Study Design**

This was a double blind, randomized, placebo-controlled multicenter trial in subject diagnosed with IBS conducted in 14 primary care and specialized gastroenterological practices in Slovenia and Croatia (Supplementary Figure S1; http://dx.doi.org/10.3168/jds.2015-10743). The study was approved by The National Medical Ethics Committee of the Republic of Slovenia (KME 28/11/11, 30.11.2011). All participants signed a written informed consent form, all participants' data were coded, and all information was kept confidential.

The study population comprised subjects aged 18 to 65 yr who met Rome III criteria (Drossman, 2006) for a diagnosis of constipation-predominant IBS with symptoms being present for >6 mo. Inclusion criteria were symptoms such as abdominal pain, bloating, and general digestive discomfort at least twice a week in the last 3 mo before inclusion and a colonoscopy, with normal results, in the last 7 yr before inclusion. Exclusion criteria at the time of enrollment were diarrhea-predominant IBS; presence of clinical signs of alarm such as rectorrhagia, fever, or recent unexplained weight loss; organic gastrointestinal diseases, including chronic inflammatory bowel disease; and systemic diseases with gastrointestinal difficulties or severe abdominal pain. Additional exclusion criteria were treatment with analgesics or antispasmodics for the previous 5 d or more, treatment with psychotropic medicines or other IBS drugs within the previous month, dietary habits that could influence the assessment of the investigational product, such as a weight-loss diet or a vegetarian diet, allergy to ingredient(s) in the investigational product, a known medical or psycho-physical condition that was, in the opinion of the investigator, in conflict with the consumption of the investigational product, or the participation in another clinical study less than 1 mo before inclusion or concurrent participation in another clinical study.

The study consisted of 3 periods: a 2-wk run-in period, a 4-wk fermented milk consumption period, and a 2-wk follow-up period. During the run-in period, the subjects were not allowed to consume probiotics or prebiotics in any form. A subgroup of 30 randomly selected subjects out of 76 study participants was subjected to detailed analyses of the fecal microbiota as presented below.

**Intervention**

All participants were randomly allocated to the synbiotic or the placebo group. The study product was a commercially available synbiotic fermented milk produced by the Mlekarna Celeia dairy plant (Petrovče, Slovenia) containing the probiotic cultures *Lactobacillus acidophilus* La-5 (on average, 1.8 × 10^7 cfu/g) and *Bifidobacterium animalis* ssp. *lactis* BB-12 (on average, 2.5 × 10^7 cfu/g; Chr. Hansen, Hørsholm, Denmark) and 2% dietary fiber Beneo Orafti Synergy1 (90% inulin, 10% oligofructose; Orafti, Mannheim, Germany). Besides the probiotic strains, the starter culture contained *Streptococcus thermophilus* (ABT-21 culture, Chr. Hansen). The placebo product was a heat-treated fermented milk without probiotic bacteria and without dietary fibers that did not noticeably differ from the synbiotic fermented milk in terms of appearance, smell, taste, or packaging.

Both the synbiotic and placebo products were packed in 180-g pots. The basic composition of both products was identical: 100 g of product contained on average 4.4 g of protein, 7.4 g of carbohydrates (of which 5.1 g were sugars), 1.5 g of fat, and 0.05 g of sodium. Both products were specifically prepared for the study and provided by the Mlekarna Celeia dairy plant. Investigators, nurses, patients, and the research team were blind to product allocation.

The patients in both groups were instructed to consume the product twice daily. All patients received a 2-wk supply of the allocated product in the first instance and instructions to receive further supplies fortnightly for 12 wk.

**Collection of Fecal Samples**

Participants collected their stool samples in sterile containers and stored them immediately after defecation at −20°C in a home freezer. Samples were transported frozen to the laboratory, stored at −20°C, and analyzed within 1 wk. Sampling was conducted in wk 2
Total Fecal Bacterial DNA Extraction

Total bacterial DNA was extracted from 1 mL of diluted (1:100) and homogenized fecal samples (as described above) using Maxwell 16 System (Promega) and Maxwell 16 Tissue DNA Purification Kit, following the protocol described previously (Bogovič Matijašič et al., 2014). The concentration of DNA was measured by using a NanoVue spectrophotometer (Fisher Scientific, Loughborough, UK). Extraction efficiency and load were verified in the real-time PCR using oligonucleotide primers for all bacteria (Eub338F and Eub518R, Supplementary Table S1; http://dx.doi.org/10.3168/jds.2015-10743).

Real-Time PCR

Samples of DNA for qPCR standard curves were prepared from pure bacterial cultures spiked into the fecal matrix as described previously (Bogovič Matijašič et al., 2014). Culture conditions and media for cultivation of *Bifidobacterium animalis* ssp. *lactis* BB-12, *Bacteroides thetaiotaomicron* DSM 2079, *Escherichia coli* K12, and *Lactobacillus acidophilus* La-5 used for standards are listed in Supplementary Table S2 (http://dx.doi.org/10.3168/jds.2015-10743).

The qPCR reactions (except for La-5 strain quantification) were accomplished in a total volume of 20 μL consisting of KAPA SYBR Fast Master Mix (2×) Universal (KapaBiosystems, Boston, MA), 0.2 μM of each of the 2 oligonucleotide primers listed in Supplementary Table S1 (http://dx.doi.org/10.3168/jds.2015-10743; Malinen et al., 2003; Bartosch et al., 2004; Rinttilä et al., 2004; Fierer et al., 2005; Songjinda et al., 2007; Malinen et al., 2003; Malinen et al., 2003; Savard et al., 2011), and 1 μL of the isolated fecal DNA (100-fold diluted). Relative quantification of the La-5 strain was conducted in a 25-μL reaction volume consisting of KAPA Probe Fast Master Mix (2×) Universal, 5 μL of the isolated fecal DNA (100-fold diluted), 0.33 μM of each of the 2 oligonucleotide primers, and 0.25 μM Zen double quencher probe (Integrated DNA Technologies Inc.). A Stratagene Mx3000P (Stratagene, La Jolla, CA) real-time PCR cycler was used. In each experiment, at least 7 dilutions (1:2) of standard DNA were included; different experiments’ standard concentrations ranged from at least 3 up to 6 log.

Except for *L. acidophilus* La-5, the results (relative abundance of bacteria) are presented as 16S rRNA copies of specific target group of bacteria per gram of feces and as a ratio (%; number of 16S rRNA copies of specific group/g: number of 16S rRNA copies of all bacteria/g). The numbers of 16S rRNA copies was approximated from the counted number of bacteria by using 16S rRNA copy number estimates according to the data available in the rrnDB database (http://rrndb.unms.med.umich.edu/). For *L. acidophilus* La-5, the results are presented as cells per gram of feces. Samples were run in duplicate and for each primer set 3 or 4 runs were conducted. No-template controls were included in each run. Except for La-5 strain quantification (probe chemistry), melting curve analysis followed
each run to verify the specificity of the PCR products obtained. For the purpose of statistical analysis, a value corresponding to half the limit of detection (LOD) was assigned to results below the LOD of qPCR assays.

**Sequencing 16S rRNA Gene Amplicons**

Sequencing of bacterial 16S rDNA amplicons (V4) of fecal samples was carried out by Illumina (San Diego, CA) MiSeq technology (Unidad de genómica, Parque Científico de Madrid, Spain), using 2 × 150 pair end run as described previously (Caporaso et al., 2012). Primers 515F/806R were used for amplification of variable region 4 (V4) of bacterial 16S rRNA gene. Library preparation followed standard Illumina MiSeq guidelines. Target coverage was 50,000 reads per DNA sample. After quality filtering, demultiplexing and primer trimming reads were processed with Mothur v1.33 (Schloss et al., 2009) following proposed pipeline (MiSec SOP, accessed December 1, 2013; Kozich et al., 2013). Operational taxonomic units (OTU) were formed using the average neighbor algorithm with 0.03 distance threshold. Bayesian classifier was used for taxonomical assignments to the genus level (Wang et al., 2007) with RDP training set 9. Species assignments were done with manual database search. Principal coordinates analysis was calculated in Mothur v1.33 (Schloss et al., 2009). Further statistical analysis was carried out in IBM SPSS Statistics for Windows (version 20; IBM Corp., Armonk, NY).

**Statistical Analyses of qPCR Data**

Results of qPCR analyzes were summarized using nonparametric descriptive statistics. Median and range (minimum and maximum) values were calculated separately for synbiotic and placebo groups and separately for the 3 sampling periods: at the end of run-in (sample 1), at the end of intervention period (sample 2), and after 1 wk of follow-up (sample 3). The difference (alteration) in qPCR data between samples 2 and 1 and that between samples 3 and 1 were calculated separately for synbiotic and placebo groups. Additionally, a ratio of L. acidophilus La-5 versus all bacteria and ratio of L. acidophilus La-5 versus Lactobacillus group were calculated and statistically analyzed. The same was done for Bifidobacterium animalis ssp. lactis.

The differences in qPCR data between synbiotic and placebo groups at the end of the run-in phase were tested using the Mann-Whitney U-test. The differences between synbiotic and placebo groups in the alteration qPCR data between samples 2 or 3 and sample 1 were also tested using the Mann-Whitney U-test. The Friedman ANOVA test was applied to test the influence of the product on qPCR data for all 3 study periods. In the post hoc analysis, the change in qPCR data from sample 1 to sample 2 and change from sample 2 to sample 3 was tested using Wilcoxon ranked sign test with Holm’s correction for multiple comparisons. This was applied separately for the synbiotic and placebo groups. Statistical significance was set at \( P < 0.05 \). All statistical analyses were performed using IBM SPSS Statistics for Windows (version 22.0; IBM Corp.).

**RESULTS**

**Effect of Consuming Synbiotic or Placebo on Fecal Microbiota**

In the feces of 30 study participants (11 from synbiotic group and 19 from placebo group), obtained first at the end of the 2-wk run-in period, second after 4 wk of consumption, and third after 1 wk of follow-up, selected groups of bacteria were quantified by qPCR. The average content of target bacteria determined by qPCR at the end of the run-in period (before consumption of synbiotic or placebo product) did not differ statistically between the synbiotic and placebo groups, except for the average quantity of DNA specific for Lactobacillus acidophilus La-5 strain, which was higher \( P = 0.042 \) in participants assigned to the synbiotic group (Table 1). The results of La-5-specific DNA were above the LOD \( (\text{average LOD} = 2.7 \times 10^4 \text{ cells/g}) \) for the initial fecal samples of 12 participants (5 from synbiotic group and 7 from placebo group).

The qPCR analyses showed statistically significant increases in certain bacterial groups during the consumption period in the synbiotic group but not in the placebo group. Subjects in the synbiotic group showed, after 4 wk of synbiotic consumption, a statistically significant increase in L. acidophilus La-5-like bacteria (Wilcoxon ranked sign test, \( P = 0.015 \); Figure 1) and B. animalis ssp. lactis (Wilcoxon ranked sign test, \( P = 0.003 \); Figure 2) compared with the values obtained at the end of the run-in period. Similar results were observed for the ratios (%) of L. acidophilus La-5 to all bacteria (Figure 1), L. acidophilus La-5 to Lactobacillus group (Figure 1), and B. animalis ssp. lactis to all bacteria (Figure 2). After 1 wk of follow-up, the values in the synbiotic group for L. acidophilus La-5 and B. animalis ssp. lactis decreased again to levels close to the initial level. Furthermore, after 4 wk of synbiotic consumption, subjects in the synbiotic group showed statistically significantly higher increases in L. acidophilus La-5-like bacteria (Mann-Whitney U-test, \( P < 0.001 \); Figure 1) and B. animalis ssp. lactis (Mann-Whitney U-test, \( P < 0.001 \); Figure 2), and a slightly
greater increase in *Lactobacillus* group (*P* = 0.0502, not significant) compared with the placebo group.

No significant changes during time or differences between the groups were observed for *Enterobacteriaceae*, *Bifidobacterium* group, or all bacteria (Supplementary Figure S2; http://dx.doi.org/10.3168/jds.2015-10743).

**Detection of Viable Probiotic Strains in Feces by RAPD Profiling**

The RAPD-PCR analysis of presumptive bifidobacteria colonies cultured from fecal samples confirmed the presence of cultivable bacteria identical or very similar to the consumed BB-12 strain in samples collected at the end of consumption period from all subjects who consumed the synbiotic product (Table 2). In some subjects, such bacteria were found even in feces collected before the consumption period, irrespective of group. The results indicate that the consumed viable BB-12 strain was well represented in the cultivable bifidobacteria fecal population in the subjects who consumed probiotic product, whereas La-5 was not, possibly because of lower survival or due to the lower ratio of consumed lactobacilli in relation to all fecal viable lactobacilli.

**Assessment of Fecal Microbiome Composition**

Samples of bacterial DNA isolated from feces of a subset of participants randomly selected from those assigned to the synbiotic group (10 subjects) and placebo group (10 subjects), taken at 3 sampling times, were also analyzed by NGS of 16S rDNA amplicons. The NGS resulted in 902,131 sequences passing quality control, with an average of 15,035 sequences per subject, which were assigned to corresponding taxonomic groups. As shown by principal coordinate analysis of 16S rDNA pyrosequencing data for 60 fecal samples, no major shift in microbial community structure was observed that was associated only with the synbiotic group (Figure 3). Small shifts were noted in all participants in both synbiotic and placebo groups. In both groups, the extent and direction of shift in treatment period and after follow-up was individual (Figure 3). Moreover, no significant changes were observed between synbiotic and placebo groups on OTU at the genus or family level (Supplementary Figure S3; http://dx.doi.org/10.3168/jds.2015-10743). However, the percentage of sequences assigned to *Strep. thermophilus* was transiently increased at the end of consumption period in both groups and decreased only slightly (not significantly) during the 1-wk follow-up (Figure 4). This observation may be associated with the use of a starter culture containing bacteria belonging to *Strep. thermophilus* in the production of the synbiotic and placebo products.

**DISCUSSION**

So far, only a few clinical studies with fermented milk products have been carried out on IBS patients (Agrawal et al., 2009; Hong et al., 2011; Veiga et al., 2014). In the present study, we examined the influence of synbiotic and placebo fermented dairy product consumption on fecal microbiota composition by metataxonomics and qPCR analyses based on bacterial 16S ribosomal gene sequences. The subjects from the present report (n = 30) were a subgroup of a larger group of patients with constipation-predominant IBS (n = 76) who were
Figure 1. Relative quantification of bacteria closely related to \textit{Lactobacillus acidophilus} La-5 in feces from the synbiotic group (n = 11) and the placebo group (n = 19). The results are presented as a logarithm of number of \textit{L. acidophilus} La-5 cells per gram of feces (top panel), as logarithm of ratio in relation to all bacteria (middle panel), and as logarithm of ratio in relation to \textit{Lactobacillus} group (bottom panel). White bars = count at the end of 2-wk run-in period; gray bars = at the end of 4-wk consumption period; hatched bars = after 1 wk of follow-up period. A value corresponding to half the limit of detection was assigned to results below the quantitative PCR limit of detection. The box and whiskers plots represent the medians and interquartile ranges; error bars represent 10th and 90th percentiles. Asterisks indicate a significant increase of \textit{L. acidophilus} La-5 during the consumption period or a significant decrease during the follow-up period ($P < 0.05$).

Figure 2. Relative quantification of bacteria closely related to \textit{Bifidobacterium animalis} ssp. \textit{lactis} in the feces from the synbiotic group (n = 11) and the placebo group (n = 19). The results are presented as a logarithm of number of 16S rDNA copies of \textit{B. animalis} ssp. \textit{lactis} per gram of feces (top panel), as logarithm of ratio in relation to all bacteria (middle panel), and as logarithm of ratio in relation to \textit{Bifidobacterium} genus (bottom panel). White bars = count at the end of 2-wk run-in period; gray bars = at the end of 4-wk consumption period; hatched bars = after 1 wk of follow-up period. A value corresponding to half the limit of detection was assigned to results below the quantitative PCR limit of detection. The box and whiskers plots represent the medians and interquartile ranges; error bars represent 10th and 90th percentiles. Asterisks indicate a significant increase of \textit{B. animalis} ssp. \textit{lactis} during the consumption period or a significant decrease during the follow-up period ($P < 0.05$).
Table 2. Prevalence of presumptive bifidobacteria (from Wilkins-Chalgren-mupirocin agar) and lactobacilli (from Rogosa agar) colonies exhibiting random amplification of polymorphic DNA (RAPD)-PCR profile identical to that of the Bifidobacterium animalis ssp. lactis BB12 or Lactobacillus acidophilus La-5 strain, respectively1

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Participant</th>
<th>Colonies of presumptive bifidobacteria/all colonies tested</th>
<th>Colonies of presumptive lactobacilli/all colonies tested</th>
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<tr>
<td>Synbiotic</td>
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<td>9/10 5/10 0/10</td>
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<td>2</td>
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1, 2, and 3 indicate the sampling times: 1 = end of run-in period; 2 = end of test products consumption period; 3 = after 1 wk of follow-up period.

2No colonies grew on the Rogosa plates.

Figure 3. Principal coordinate analysis of metataxonomics data for 60 fecal samples obtained from subjects in the synbiotic (n = 10; left) and placebo (n = 10; right) groups at 3 sampling points; that is, before consumption period, at the end of consumption period, and after 1 wk of follow-up period. The first 2 dimensions are shown, and ordination is split into 2 plots. Dots from the same individual are linked with a line. Arrows indicate chronological sampling points. Color version available online.
included in a double-blind, placebo controlled, randomized multicentric study (NCT02391220; https://clinicaltrials.gov/ct2/show/NCT02391220) aimed at assessing the effect of a synbiotic fermented milk on health-related quality of life (QoL) and IBS symptoms (Alenka Šmid, University of Ljubljana, Faculty of Pharmacy, Slovenia; Luka Strniša, University Medical Centre Ljubljana, Ljubljana, Slovenia; Katarina Bajc and Dunja Vujić-Podlipec, Clinres Farmacija, Ljubljana, Slovenia; Bojana Bogović Matijašić; Irena Rogelj; unpublished data). The primary outcome was an improvement in overall IBS-QoL score; other outcomes included frequency and severity of pain, bloating severity, bowel movement frequency, and satisfaction. The sample size calculation for the overall study was based on previous evaluations of the IBS-QoL questionnaire in patients with IBS, taking into account a goal to demonstrate a 10-point difference in the average value for the total number of points and a test power of 80%. The size of the subgroup subjected to the study of fecal microbiota composition was based on the feasibility of microbiological and molecular analyses, considering research capacity and financial resources.

The results of RAPD analysis of presumptive bifidobacteria indicated the presence of BB-12-like bacteria in the microbiota of tested subjects even before the trial, although the participants of the study were asked to avoid consumption of any fermented milk products or other type of products containing probiotics or prebiotics during the 2-wk run-in period. Alander et al. (2001) and Matto et al. (2006) explained similar observations in 2 studies on healthy volunteers consuming...
BB-12 probiotic strain by the fact that this organism is commonly used in probiotic foods and may persist in certain subjects for a few weeks. It is also possible that these subjects accidentally consumed a product containing BB-12 during the run-in period. However, the prevalence of BB-12-like bacteria after consumption was much higher in the synbiotic group and in the samples taken after probiotic consumption than before it; therefore, we concluded that the probiotic strain identified in the participants of our study mainly originated in the consumed synbiotic product. Moreover, these results confirm the survival of BB-12 strain during passage through the gastrointestinal tract. The colonies identical to the La-5 strain were rarely detected and could be attributed to probiotic product consumption in only one subject.

The use of a L. acidophilus La-5-specific probe enabled qPCR detection of this strain or very closely related strains in 12 participants (40%; average LOD = 2.7 × 10^4 cells/g) in both groups. This observation may be explained by the fact that strain La-5 is widely used in various probiotic products and might have become established in the gut of some study participants. In a similar clinical trial with commercial yogurt containing these 2 strains, L. acidophilus La-5 was also detected by qPCR, using the same primers and probe, in the feces of 7% of participants (LOD 3.3 × 10^4 cfu/g) at the baseline sampling (Savard et al., 2011). In the same study, the researchers detected B. animalis ssp. lactis in 56% of subjects (LOD 2.5 × 10^4 cfu/g), whereas Palaria et al. (2012) reported the absence of this subspecies in baseline feces. This difference could be also due to the probe and the specific qPCR assay used in the latter study, which might be more specific for the BB-12 strain. Direct comparison of the amounts of La-5 and BB-12 strains (or B. animalis ssp. lactis) among different studies showing the presence of these strains in feces before any intervention, however, is not possible because of different durations of run-in period and different methods for quantification or various limits of detection.

The greater increase in amounts and proportion of L. acidophilus La-5 and B. animalis ssp. lactis in feces of the synbiotic group compared with the placebo group established by qPCR confirms the short-term effect of both ingested strains on the fecal microbiomes. Although the qPCR quantification of La-5 strain was specific because of the availability of previously designed strain-specific oligonucleotide PCR primers (Savard et al., 2011), BB-12 strain-specific oligonucleotides have not been described in the literature so far, but Bifidobacterium animalis ssp. lactis subspecies-specific PCR were found to be specific enough for strain BB-12 quantification, as, in general, this subspecies is not abundant in the indigenous fecal microbiota (Savard et al., 2011; Palaria et al., 2012). Moreover, even if the qPCR assays were not absolutely selective for these 2 strains, it could be expected that considerable changes in the fecal concentration would be successfully detected.

Although the relative abundance of the ingested probiotic strains in the total fecal microbiota was very low; that is, <1% B. animalis ssp. lactis (median ratio <0.1%) and <0.1% L. acidophilus La-5 (median ratio <0.01%), it is important to consider that these bacteria can act in the stomach and small intestine where their relative abundance is much higher (Turroni et al., 2014).

The observed persistence of L. acidophilus La-5 and B. animalis ssp. lactis BB-12 varies among studies carried out with different matrices, times of consumption, limits of detection, and other factors. Palaria et al. (2012), for instance, reported that BB-12 survived passage through the gastrointestinal tract but did not persist in the gut: the strain could not be detected in feces after a 2-wk washout period. Saxelin et al. (2010) observed that the median excretion time of BB-12 was 17 d when it was consumed in yogurt, but only 7 d when consumed in the form of capsules. The BB-12 strain consumed in the form of lyophilized powder was also readily detected after 1 wk of washout by RAPD analysis of the colonies, but not after 2 wk (Alander et al., 2001). In 1 out of 14 healthy subjects consuming probiotic yogurt, BB-12 was found in feces 19 d after the end of consumption as well as in the colonic mucosa; in the other subjects, it was washed out more quickly (Matto et al., 2006). In our study, the abundance of L. acidophilus La-5 and B. animalis ssp. lactis-specific DNA decreased during the 1-wk follow-up period to the levels close to initial levels, indicating only transient colonization of the applied probiotic strains. This is in accordance with previous studies.

The Enterobacteriaceae group was selected for qPCR analysis because this family comprises several potentially pathogenic bacteria with the ability to cause inflammation, which can contribute to the development of IBS. Furthermore, some previous studies reported the increased abundance of enterobacteria in diarrhea-predominant IBS patients (Carroll et al., 2012) and an increase in some members of Enterobacteriaceae (Escherichia) in IBS patients in general, including diarrhea-predominant, constipation-predominant, and alternating IBS (Rajilić-Stojanović et al., 2011). The increase of B. animalis ssp. lactis in the fecal microbiomes of healthy subjects consuming yogurt with La-5 and BB-12 strains, however, was reported to be negatively correlated with the enterobacteria content (Filteau et al., 2013). In another study on 46 healthy subjects consuming a symbiotic fermented milk product
containing yogurt starter and BB-12 strain, a decrease in enterobacteria counts was observed in a subgroup of subjects with higher initial enterobacteria counts (>5.5 log10 cfu/g; Palaria et al., 2012). Similar results were observed in the placebo group, indicating that consumption of the probiotic was not responsible for this effect. Nevertheless, in our study, as in some other previous studies (Savard et al., 2011), the abundance of enterobacteria was not affected by synbiotic or placebo consumption.

Based on qPCR quantification, the bacteria consumed by the subjects from either group did not significantly influence the total amount of lactobacilli or bifidobacteria in the feces, whereas the amount and proportion of La-5 and B. animalis ssp. lactis increased in subjects in the synbiotic group. These results are in accordance with reports of the previous clinical study on 58 healthy subjects who consumed, for 4 wk, yogurt with the La-5 and BB-12 strains and Strep. thermophilus as starter culture or acidified milk as placebo (Filteau et al., 2013). In the study of Palaria et al. (2012), B. animalis ssp. lactis BB-12 was also increased after BB-12 strain ingestion, as established by strain-specific qPCR in which only viable cells were quantified, whereas the increase of total bifidobacteria was significant only for the subgroup of subjects with lower baseline bifidobacteria load (<10⁸ cfu/g) and who consumed the synbiotic product. In a study on 74 IBS subjects given fermented milk that contained 2 Lactobacillus probiotic strains (Lactobacillus HY7801, Lactobacillus brevis HY7401) and Bifidobacterium longum HY8004, counts of the lactobacilli but not the bifidobacteria were increased significantly in feces of IBS patients receiving treatment (Hong et al., 2011). The level of lactobacilli was increased during the consumption of synbiotic product with L. acidophilus NCFM and cellubiose (van Zanten et al., 2014).

Similar to results of the qPCR analysis, 16S rDNA-based fecal microbiome pyrosequencing did not reveal significant shifts in the average abundance of total lactobacilli or bifidobacteria in the feces of study participants in the placebo or synbiotic groups. The differences in the abundance of L. acidophilus La-5 and B. animalis ssp. lactis in the synbiotic group observed by qPCR could not be confirmed by NGS. Interestingly, an increase in the abundance of Strep. thermophilus, a species contained in the starter culture used for the production of test and placebo products, was detected in both groups. The most probable reason for this observation is methodological. In other words, due to the nature of the 16S rRNA gene and short amplicon length in high-throughput pyrosequencing, detection is not species-specific for all OTU, even less subspecies- or strain-specific. Related bacteria can be included in the same OTU and mask the strain-specific signal. This was not, however, the case with Strep. thermophilus-specific OTU. Furthermore, as the abundance of Strep. thermophilus in the product was higher compared with that of each probiotic culture (30-fold higher, on average), the greater detection of this species could simply be a matter of detection limits for these bacterial groups. The Strep. thermophilus starter culture, however, is generally not expected to survive gastrointestinal passage in high numbers; starter culture strains are selected on the basis of technological properties not probiotic or health-promoting properties, including good survival in the gut. We should also consider that the 16S rDNA-based microbiome pyrosequencing does not distinguish between DNA originating from viable cells and DNA released from inactivated or dead cells; abundance is estimated based on the total amount of DNA.

Principal coordinate analysis has been successfully used in some previous studies for the identification of the changes in the microbiome. In contrast to the recent report of Unno et al. (2015), who observed a consistent fecal microbial community shift in 5 of 6 individuals consuming a synbiotic fermented milk product containing lactobacilli, bifidobacteria, and Strep. thermophilus, the shifts observed in our study were not consistent and not associated with treatment. van Zanten et al. (2014) analyzed the microbiota of 18 healthy volunteers consuming placebo or probiotic powder with L. acidophilus NCFM and cellubiose. From the results of principal coordinate analysis, based on weighted UNIFRAC distance matrix, van Zanten et al. (2014) concluded that the pattern of each volunteer was highly individual and did not cluster according to treatment. Highly individual changes in gut microbiota in response to probiotic intake were also observed in 18 subjects consuming 6 commercially available probiotics in capsules containing either Bifidobacterium or Lactobacillus strains (Kim et al., 2013). The absence of significant changes in the overall structure of fecal microbiome (on the class, order, or family level) was not surprising, because the amount of ingested probiotic bacteria was relatively low compared with the entire gut microbiota. Similar results were reported in some previous studies dealing with the effects of probiotics on the microbiomes (Kim et al., 2013; van Zanten et al., 2014; Veiga et al., 2014). In different studies, changes in particular groups of microorganisms at the genus or species level as a result of probiotic or synbiotic treatment were observed by 16S rRNA profiling of fecal microorganisms. Increases of the genera Lactobacillus, Bifidobacterium, Collinsella, and Eubacterium levels and a decrease in Dialister were observed in the feces of subjects consuming synbiotic product with Lactobacillus acidophilus NCFM and cellubiose (van
Zanten et al., 2014). Commercial *Lactobacillus* or *Bifidobacterium* strains were given to 18 healthy subjects for 8 wk, and 24 OTU assigned to indigenous species were changed ≥10-fold (Kim et al., 2013). In contrast to the results of our study, the composition of fecal microbiomes of 6 healthy females consuming symbiotic fermented milk containing 4 probiotic strains belonging to *Lactobacillus* or *Bifidobacterium* was changed in all subjects at the phylum level (Unno et al., 2015). The observed increase of *Bacteroidetes* was attributed to the increase in the abundance of *Bacteroidaceae* or *Prevotellaceae*, whereas the decrease of *Firmicutes* to the decrease of *Ruminococcaceae* and *Lachnospiraceae* family members. In contrast to our approach based on 16S rDNA profiling, Veiga et al. (2014) applied a whole-genome metagenomic sequencing approach, which enables more accurate identification of microbiome composition at the species level as well as determination of metabolic capacity. In 13 IBS patients treated with a fermented milk product containing 5 different strains, including starter cultures and probiotic *B. animalis* ssp. *lactis* CNCM I-2497, and increased level of potential short-chain fatty acid producers and decreased level of an opportunistic pathogen *Bilophila wadsworthia* were found.

Several recent studies have demonstrated that although particular probiotics had little effect on fecal microbiota composition, they could affect gene expression of microbiota and metabolic products. For instance, a 4-wk consumption of fermented milk product containing 4 starter culture strains and probiotic strain *B. animalis* ssp. *lactis* CNCM I-2494 induced some changes in human fecal transcriptome in gnotobiotic mice harboring a 15-species model human gut microbiota (McNulty et al., 2011). Many of the genes affected were those related to carbohydrate metabolism. The transcriptional response of the gut microbiome of 12 healthy elderly subjects was found to be modulated by treatment with probiotic strain *Lactobacillus rhamnosus* GG ATCC 53103 (LGG; Elo-Fadrosh et al., 2015); the expression of genes involved in flagellar motility, chemotaxis, and adhesion of bacteria was increased. These recent examples demonstrate that certain probiotics are able to alter physiologic systems of the human host and promote a particular functional effect. Alleviation of IBS symptoms by probiotic consumption has been demonstrated several times previously, but evidence of the functional effects was not available. Hong et al. (2011), however, reported a trial on 74 patients with IBS who received a multistrain probiotic fermented milk product or placebo for 8 wk. In a subset of patients, alterations in blood glucose, lactate, and tyrosine were normalized by probiotic treatment, indicating that energy homeostasis and liver function might be improved. More studies including transcriptomics and metabolomics approaches are needed to further reveal the effects of probiotic, prebiotic, and symbiotic products on patients with IBS.

**CONCLUSIONS**

Daily consumption of fermented milk with probiotic strains *Lactobacillus acidophilus* La-5 (≥6.5 × 10⁹ cfu) and *Bifidobacterium animalis* ssp. *lactis* BB-12 (≥9 × 10⁹ cfu) and dietary fiber (90% inulin, 10% oligofructose) had a short-term effect on the amount and proportion of La-5-like strains and *B. animalis* ssp. *lactis* in the fecal microbiome of IBS patients. The abundance or proportion of *Enterobacteriaceae, Lactobacillus, Bifidobacterium*, or all bacteria was not significantly affected by consumption of the symbiotic or placebo product. The global profile of the fecal microbiota of patients was not disturbed by any treatment. However, the percentage of sequences assigned to *Streptococcus thermophiles*, a species contained in the starter culture used for the production of symbiotic and placebo products, was transiently increased.

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