Effects of synbiotic fermented milk containing *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* ssp. *lactis* BB-12 on the fecal microbiota of adults with irritable bowel syndrome: A randomized double-blind, placebo-controlled trial

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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 *Bifidobacterium 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

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modifications, consumption of probiotics and prebiotics as supportive therapy (Sayuk and Gyawali, 2015).

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 microbiome 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 rDNA 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 Orafit Synergy1 (90% inulin, 10% oligofructose; Orafit, 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

(at the end of run-in), 6 (at the end of intervention period), and 7 (after 1 wk of follow-up) of the experiment.

RAPD Profiling

Fecal samples of 20 randomly selected subjects (8 from the synbiotic group and 12 from the placebo group) collected at 3 sampling times were tested by RAPD profiling for the presence of viable strains resembling BB-12 and La-5. Each stool sample (0.1 g) was homogenized in 9.9 g of anaerobic diluent (1 g/L peptone, 2 g/L porcine gelatin, 8.5 g/L NaCl, and 0.557 g/L L-cysteine hydrochloride monohydrate) and diluted. Then, 100 μ L of sample was spread on Rogosa agar plates (Merck, Darmstadt, Germany) for lactobacilli and Wilkins-Chalgren medium (Oxoid, Basingstoke, UK) according to Ferraris et al. (2010) with addition of mupirocin (50 μ L/mL; AppliChem, Darmstadt, Germany) for bifidobacteria, respectively. Plates were incubated anaerobically at 37°C for 72 h.

Ten colonies from Rogosa and 10 colonies from Wilkins-Chalgren-mupirocin plates were randomly selected and transferred to a nucleic acid extraction kit SmartHelix EZextract (Institute of Physical Biology, Ljubljana, Slovenia). The DNA extracts were then further submitted to RAPD-PCR amplification carried out using M13 primer (5'-GAGGGTGGCGGTTCT-3') for lactobacilli and 1254 primer (5'-CCGCAGCCAA-3') for bifidobacteria (Akopyanz et al., 1992; Marzotto et al., 2006). The oligonucleotides M13 and 1254 were chosen based on previously established (results not shown) good discrimination potential among representatives of closely related lactobacilli species and bifidobacteria, respectively and on literature data (Torriani et al., 1999; Tušar et al., 2014). The PCR reactions were performed in 20- μ L reaction mixtures containing appropriate amounts of 5 $\times$ GoTaq flexi (Promega, Madison, WI), MgCl₂ (25 mM; Promega), oligonucleotides (Integrated DNA Technologies, Coralville, IA), dNTP (10 mM; Thermo Scientific, Waltham, MA), GoTaq DNA polymerase (5 U/ μ L; Promega), and 1 μ L of DNA extracts, following previously described PCR programs (Torriani et al., 1999). Amplification products were analyzed by electrophoresis in 1.8% gels with Tris-acetate-EDTA buffer and stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA).

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 Matijašić 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 Matijašić 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 μ L consisting of KAPA SYBR Fast Master Mix (2 $\times$) Universal (KapaBiosystems, Boston, MA), 0.2 μ M 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; Savard et al., 2011), and 1 μ L of the isolated fecal DNA (100-fold diluted). Relative quantification of the La-5 strain was conducted in a 25- μ L reaction volume consisting of KAPA Probe Fast Master Mix (2 $\times$) Universal, 5 μ L of the isolated fecal DNA (100-fold diluted), 0.33 μ M of each of the 2 oligonucleotide primers, and 0.25 μ M Zen double quencher probe (Integrated DNA Technologies Inc.). A Stratagene Mx3000P (Stratagene, La Jolla, CA) real-time PCR cyclor 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 rDNA copies of specific target group of bacteria per gram of feces and as a ratio (%; number of 16S rDNA copies of specific group/g: number of 16S rDNA copies of all bacteria/g). The numbers of 16S rDNA copies was approximated from the counted number of bacteria by using 16S rDNA copy number estimates according to the data available in the *rrn*DB database (<http://rrndb.umms.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 (MiSeq 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 Fried-

man 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 (average LOD = 2.7×10^4 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

Table 1. The content of bacteria [median (range)] of number of 16S rDNA copies per gram or cells per gram (for *Lactobacillus acidophilus* La-5 only) in the fecal samples of 30 participants in the study randomly assigned either to the synbiotic group or placebo group, determined at the end of run-in period

Species/group	Synbiotic (n = 11)	Placebo (n = 19)	P-value ¹
<i>Lactobacillus acidophilus</i> La-5	2.5×10^4 (1.2×10^4 to 4.1×10^7) ²	1.2×10^4 (3.4×10^3 to 6.0×10^7) ²	0.042*
<i>Bifidobacterium animalis</i> ssp. <i>lactis</i>	4.2×10^7 (2.3×10^5 to 9.0×10^8) ²	3.1×10^6 (2.3×10^5 to 2.8×10^9) ²	0.714
<i>Enterobacteriaceae</i>	1.0×10^8 (2.9×10^6 to 1.3×10^9)	1.1×10^8 (3.8×10^6 to 4.3×10^{10})	0.451
<i>Bifidobacterium</i> genus	2.4×10^{10} (8.4×10^6 to 8.3×10^{10})	7.0×10^9 (2.5×10^7 to 4.2×10^{10})	0.204
<i>Lactobacillus</i> group	1.2×10^8 (1.1×10^7 to 7.9×10^8)	1.4×10^8 (5.1×10^6 to 4.3×10^9)	0.715
All bacteria	3.8×10^{12} (1.0×10^{12} to 1.1×10^{13})	2.9×10^{12} (6.9×10^{11} to 1.4×10^{13})	0.505

¹Mann-Whitney U-test (synbiotic vs. placebo group).

²The lower value in the range corresponds to one-half of the limit of detection (LOD) for results below the LOD of quantitative PCR assays for the purpose of statistical analysis.

* $P < 0.05$.

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*, *Lactobacillus* 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 *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 *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 *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 *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 *Bifidobacterium animalis* ssp. *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 *B. animalis* ssp. *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 *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 *B. animalis* ssp. *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

Treatment group	Participant	Colonies of presumptive bifidobacteria/all colonies tested			Colonies of presumptive lactobacilli/all colonies tested		
		1	2	3	1	2	3
Synbiotic	1	9/10	5/10	0/10	10/10	10/10	0/10
	2	4/10	10/10	8/10	0/10	0/10	0/10
	3	7/10	8/10	3/10	0/10	0/10	0/10
	4	0/10	2/10	4/10	0/10	0/10	0/10
	5	0/10	6/10	7/10	0/10	0/10	0/10
	6	0/10	9/10	9/10	0/10	6/10	1/10
	7	0/10	10/10	7/10	0/10	0/10	0/10
	8	10/10	9/10	2/10	2/10	10/10	10/10
Placebo	1	0/10	0/10	0/10	0/10	0/10	0/10
	2	5/10	0/10	10/10	0/10	— ²	1/10
	3	0/10	0/10	0/10	0/10	0/10	0/10
	4	0/10	0/10	0/10	0/10	0/10	0/10
	5	0/10	0/10	0/10	0/10	0/10	0/10
	6	3/10	0/10	0/10	0/10	0/10	0/10
	7	10/10	4/10	3/10	0/10	0/10	4/10
	8	0/10	0/10	0/10	0/10	0/10	0/10
	9	0/10	0/10	0/10	0/10	0/10	0/10
	10	10/10	10/10	10/10	0/10	0/10	0/10
	11	0/10	0/10	0/10	0/10	0/10	0/10
	12	0/10	0/10	0/10	0/10	0/10	0/10

¹1, 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.

Figure 4. The average ratio (%) of *Streptococcus thermophilus*-specific sequences in relation to all sequences obtained by next-generation of 16S rDNA amplicons of the fecal microbiomes of 20 patients with irritable bowel syndrome (10 subjects from the synbiotic group and 10 from the placebo group) analyzed at 3 sampling times: 1 = end of 2-wk run-in period; 2 = end of 4-wk consumption period; 3 = after 1 wk of follow-up period. The box and whiskers plots represent the medians and interquartile ranges; error bars represent 10th and 90th percentiles. Asterisks indicate a significantly higher ratio of *Strep. thermophilus*-specific sequences (%) in feces microbiomes of both groups at the end of the 4-wk consumption period compared with that collected at the end of the 2-wk run-in period.

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 Vujič-Podlipec, Clinres Farmacija, Ljubljana, Slovenia; Bojana Bogovič 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 question-

naire 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 abun-

dant 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 synbiotic 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 ($>5.5 \log_{10}$ 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 ($<10^8$ 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 cellobiose (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 cellobiose. 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 microbiomes. 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 cellobiose (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 synbiotic 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-Fadrosch 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 synbiotic products on patients with IBS.

CONCLUSIONS

Daily consumption of fermented milk with probiotic strains *Lactobacillus acidophilus* La-5 ($\geq 6.5 \times 10^9$ cfu) and *Bifidobacterium animalis* ssp. *lactis* BB-12 ($\geq 9 \times 10^9$ 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 synbiotic 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 thermophilus*, a species contained in the starter culture used for the production of synbiotic and placebo products, was transiently increased.

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REFERENCES

- Agrawal, A., L. A. Houghton, J. Morris, B. Reilly, D. Guyonnet, N. G. Feuillerat, A. Schlumberger, S. Jakob, and P. J. Whorwell. 2009. Clinical trial: The effects of a fermented milk product containing *Bifidobacterium lactis* DN-173 010 on abdominal distension and gastrointestinal transit in irritable bowel syndrome with constipation. *Aliment. Pharmacol. Ther.* 29:104–114. <http://dx.doi.org/10.1111/j.1365-2036.2008.03853.x>.
- Akopyanz, N., N. O. Bukanov, T. U. Westblom, S. Kresovich, and D. E. Berg. 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res.* 20:5137–5142. <http://dx.doi.org/10.1093/nar/20.19.5137>.
- Alander, M., J. Matto, W. Kneifel, M. Johansson, B. Kogler, R. Crittenden, T. Mattila-Sandholm, and M. Saarela. 2001. Effect of galacto-oligosaccharide supplementation on human faecal microflora and on survival and persistence of *Bifidobacterium lactis* Bb-12 in the gastrointestinal tract. *Int. Dairy J.* 11:817–825. [http://dx.doi.org/10.1016/S0958-6946\(01\)00100-5](http://dx.doi.org/10.1016/S0958-6946(01)00100-5).
- Andriulli, A., M. Neri, C. Loguercio, N. Terreni, A. Merla, M. P. Cardarella, A. Federico, F. Chilovi, G. L. Milandri, M. De Bona, S. Cavenati, S. Gullini, R. Abbiati, N. Garbagna, R. Cerutti, and

- E. Grossi. 2008. Clinical trial on the efficacy of a new symbiotic formulation, Flortec, in patients with irritable bowel syndrome—A multicenter, randomized study. *J. Clin. Gastroenterol.* 42:S218–S223.
- Bartosch, S., A. Fite, G. T. Macfarlane, and M. E. T. McMurdo. 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl. Environ. Microbiol.* 70:3575–3581. <http://dx.doi.org/10.1128/AEM.70.6.3575-3581.2004>.
- Benno, P., R. Befrits, E. Norin, A. Berstad, A. L. Dahlgren, T. Midtvedt, and P. M. Hellström. 2015. Is irritable bowel syndrome a dysbiotic bowel syndrome? *Microb. Ecol. Health Dis.* 26:27637 <http://dx.doi.org/10.3402/mehd.v26.27637>.
- Bogović Matijašić, B., T. Obermajer, L. Lipoglavšek, I. Grabnar, G. Avguštin, and I. Rogelj. 2014. Association of dietary type with fecal microbiota in vegetarians and omnivores in Slovenia. *Eur. J. Nutr.* 53:1051–1064. <http://dx.doi.org/10.1007/s00394-013-0607-6>.
- Canavan, C., J. West, and T. Card. 2014. The epidemiology of irritable bowel syndrome. *Clin. Epidemiol.* 6:71–80. <http://dx.doi.org/10.2147/CLEP.S40245>.
- Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S. M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J. A. Gilbert, G. Smith, and R. Knight. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6:1621–1624. <http://dx.doi.org/10.1038/ismej.2012.8>.
- Cappello, C., F. Tremolaterra, A. Pascariello, C. Ciacci, and P. Iovino. 2013. A randomised clinical trial (RCT) of a symbiotic mixture in patients with irritable bowel syndrome (IBS): Effects on symptoms, colonic transit and quality of life. *Int. J. Colorectal Dis.* 28:349–358. <http://dx.doi.org/10.1007/s00384-012-1552-1>.
- Carroll, I. M., T. Ringel-Kulka, J. P. Siddle, and Y. Ringel. 2012. Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. *Neurogastroenterol. Motil.* 24:521–530. <http://dx.doi.org/10.1111/j.1365-2982.2012.01891.x>.
- Derrien, M., and J. E. T. H. Vlieg. 2015. Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends Microbiol.* 23:354–366. <http://dx.doi.org/10.1016/j.tim.2015.03.002>.
- Drossman, D. A. 2006. The functional gastrointestinal disorders and the Rome III process. *Gastroenterology* 130:1377–1390. <http://dx.doi.org/10.1053/j.gastro.2006.03.008>.
- Eloe-Fadros, E. A., A. Brady, J. Crabtree, E. F. Drabek, B. Ma, A. Mahurkar, J. Ravel, M. Haverkamp, A. M. Fiorino, C. Botelho, I. Andreyeva, P. L. Hibberd, and C. M. Fraser. 2015. Functional dynamics of the gut microbiome in elderly people during probiotic consumption. *Mbio* 6:e00231 <http://dx.doi.org/10.1128/mBio.00231-15>.
- Ferraris, L., J. Aires, A. J. Waligora-Dupriet, and M. J. Butel. 2010. New selective medium for selection of bifidobacteria from human feces. *Anaerobe* 16:469–471. <http://dx.doi.org/10.1016/j.anaerobe.2010.03.008>.
- Fierer, N., J. A. Jackson, R. Vilgalys, and R. B. Jackson. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* 71:4117–4120. <http://dx.doi.org/10.1128/AEM.71.7.4117-4120.2005>.
- Filteau, M., S. Matamoros, P. Savard, and D. Roy. 2013. Molecular monitoring of fecal microbiota in healthy adults following probiotic yogurt intake. *PharmaNutrition* 1:123–129. <http://dx.doi.org/10.1016/j.phanu.2013.05.002>.
- FAO-WHO (Food and Agricultural Organization of the United Nations and World Health Organization). 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Accessed Mar. 14, 2016. http://www.mesanders.com/probio_report.pdf.
- García-Albiach, R., M. Jose, P. de Felipe, S. Angulo, M. I. Morosini, D. Bravo, F. Baquero, and R. del Campo. 2008. Molecular analysis of yogurt containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in human intestinal microbiota. *Am. J. Clin. Nutr.* 87:91–96.
- Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota—Introducing the concept of prebiotics. *J. Nutr.* 125:1401–1412.
- Gibson, G. R., K. P. Scott, R. A. Rastall, K. M. Tuohy, A. Hotchkiss, A. Dubert-Ferrandon, M. Gareau, E. F. Murphy, D. Saulnier, G. Loh, S. Macfarlane, N. Delzenne, Y. Ringel, G. Koziánowski, R. Dickman, I. Lenoir-Wijnkoop, C. Walker, and R. Buddington. 2010. Dietary prebiotics: Current status and new definition. *Food Sci. Technol. Bull. Funct. Foods* 7:1–19. <http://dx.doi.org/10.1616/1476-2137.15880>.
- Hill, C., F. Guarner, G. Reid, G. R. Gibson, D. J. Merenstein, B. Pot, L. Morelli, R. B. Canani, H. J. Flint, S. Salminen, P. C. Calder, and M. E. Sanders. 2014. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11:506–514. <http://dx.doi.org/10.1038/nrgastro.2014.66>.
- Hong, Y. S., K. S. Hong, M. H. Park, Y. T. Ahn, J. H. Lee, C. S. Huh, J. Lee, I. K. Kim, G. S. Hwang, and J. S. Kim. 2011. Metabonomic understanding of probiotic effects in humans with irritable bowel syndrome. *J. Clin. Gastroenterol.* 45:415–425. <http://dx.doi.org/10.1097/MCG.0b013e3182077f6c>.
- Kajander, K., E. Myllyluoma, M. Rajilic-Stojanovic, S. Kyrönpallo, M. Rasmussen, S. Jarvenpää, E. G. Zoetendal, W. M. De Vos, H. Vapaatalo, and R. Korpela. 2008. Clinical trial: Multispecies probiotic supplementation alleviates the symptoms of irritable bowel syndrome and stabilizes intestinal microbiota. *Aliment. Pharmacol. Ther.* 27:48–57. <http://dx.doi.org/10.1111/j.1365-2036.2007.03542.x>.
- Kim, S. W., W. Suda, S. Kim, K. Oshima, S. Fukuda, H. Ohno, H. Morita, and M. Hattori. 2013. Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. *DNA Res.* 20:241–253. <http://dx.doi.org/10.1093/dnares/dst006>.
- König, J., and R. J. Brummer. 2014. Alteration of the intestinal microbiota as a cause of and a potential therapeutic option in irritable bowel syndrome. *Benef. Microbes* 5:247–261. <http://dx.doi.org/10.3920/BM2013.0033>.
- Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79:5112–5120. <http://dx.doi.org/10.1128/AEM.01043-13>.
- Longstreth, G. F., W. G. Thompson, W. D. Chey, L. A. Houghton, F. Mearin, and R. C. Spiller. 2006. Functional bowel disorders. *Gastroenterology* 130:1480–1491. <http://dx.doi.org/10.1053/j.gastro.2005.11.061>.
- Malinen, E., A. Kassinen, T. Rinttilä, and A. Palva. 2003. Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology* 149:269–277. <http://dx.doi.org/10.1099/mic.0.25975-0>.
- Malinen, E., L. Krogius-Kurikka, A. Lyra, J. Nikkila, A. Jaaskelainen, T. Rinttilä, T. Vilpponen-Salmela, A. J. von Wright, and A. Palva. 2010. Association of symptoms with gastrointestinal microbiota in irritable bowel syndrome. *World J. Gastroenterol.* 16:4532–4540. <http://dx.doi.org/10.3748/wjg.v16.i36.4532>.
- Marzotto, M., C. Maffei, T. Paternoster, R. Ferrario, L. Rizzotti, M. Pellegrino, F. Dellaglio, and S. Torriani. 2006. *Lactobacillus paracasei* A survives gastrointestinal passage and affects the fecal microbiota of healthy infants. *Res. Microbiol.* 157:857–866. <http://dx.doi.org/10.1016/j.resmic.2006.06.007>.
- Matto, J., R. Fonden, T. Tolvanen, A. von Wright, T. Vilpponen-Salmela, R. Satokari, and M. Saarela. 2006. Intestinal survival and persistence of probiotic *Lactobacillus* and *Bifidobacterium* strains administered in triple-strain yoghurt. *Int. Dairy J.* 16:1174–1180. <http://dx.doi.org/10.1016/j.idairyj.2005.10.007>.
- McNulty, N. P., T. Yatsunenko, A. Hsiao, J. J. Faith, B. D. Muegge, A. L. Goodman, B. Henrissat, R. Oozeer, S. Cools-Portier, G.

- Gobert, C. Chervaux, D. Knights, C. A. Lozupone, R. Knight, A. E. Duncan, J. R. Bain, M. J. Muehlbauer, C. B. Newgard, A. C. Heath, and J. I. Gordon. 2011. The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. *Sci. Transl. Med.* 3:106ra106 <http://dx.doi.org/10.1126/scitranslmed.3002701>.
- Ng, S. C., E. F. C. Lam, T. T. Y. Lam, Y. W. Chan, W. D. Law, P. C. H. Tse, M. A. Kamm, J. J. Y. Sung, F. K. L. Chan, and J. C. Y. Wu. 2013. Effect of probiotic bacteria on the intestinal microbiota in irritable bowel syndrome. *J. Gastroenterol. Hepatol.* 28:1624–1631. <http://dx.doi.org/10.1111/jgh.12306>.
- Palaria, A., I. Johnson-Kanda, and D. J. O'Sullivan. 2012. Effect of a synbiotic yogurt on levels of fecal bifidobacteria, clostridia, and enterobacteria. *Appl. Environ. Microbiol.* 78:933–940. <http://dx.doi.org/10.1128/AEM.05848-11>.
- Quigley, E. M. M. 2011. Commensal bacteria: The link between IBS and IBD? *Curr. Opin. Clin. Nutr. Metab. Care* 14:497–503. <http://dx.doi.org/10.1097/MCO.0b013e328348c033>.
- Rajilić-Stojanović, M., E. Biagi, H. Heilig, K. Kajander, R. A. Kekkonen, S. Tims, and W. M. de Vos. 2011. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology* 141:1792–1801. <http://dx.doi.org/10.1053/j.gastro.2011.07.043>.
- Rinttilä, T., A. Kassinen, E. Malinen, L. Krogius, and A. Palva. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J. Appl. Microbiol.* 97:1166–1177. <http://dx.doi.org/10.1111/j.1365-2672.2004.02409.x>.
- Roberts, L. M., D. McCahon, R. Holder, S. Wilson, and F. D. R. Hobbs. 2013. A randomised controlled trial of a probiotic 'functional food' in the management of irritable bowel syndrome. *BMC Gastroenterol.* 13:45 <http://dx.doi.org/10.1186/1471-230X-13-45>.
- Savard, P., B. Lamarche, M. E. Paradis, H. Thiboutot, E. Laurin, and D. Roy. 2011. Impact of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus acidophilus* LA-5-containing yoghurt, on fecal bacterial counts of healthy adults. *Int. J. Food Microbiol.* 149:50–57. <http://dx.doi.org/10.1016/j.ijfoodmicro.2010.12.026>.
- Saxelin, M., A. Lassig, H. Karjalainen, S. Tynkkynen, A. Surakka, H. Vapaatalo, S. Jarvenpää, R. Korpela, M. Mutanen, and K. Hatakka. 2010. Persistence of probiotic strains in the gastrointestinal tract when administered as capsules, yoghurt, or cheese. *Int. J. Food Microbiol.* 144:293–300. <http://dx.doi.org/10.1016/j.ijfoodmicro.2010.10.009>.
- Sayuk, G. S., and C. P. Gyawali. 2015. Irritable bowel syndrome: Modern concepts and management options. *Am. J. Med.* 128:817–827. <http://dx.doi.org/10.1016/j.amjmed.2015.01.036>.
- Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75:7537–7541. <http://dx.doi.org/10.1128/AEM.01541-09>.
- Songjinda, P., J. Nakayama, A. Tateyama, S. Tanaka, M. Tsubouchi, C. Kiyohara, T. Shirakawa, and K. Sonomoto. 2007. Differences in developing intestinal microbiota between allergic and non-allergic infants: A pilot study in Japan. *Biosci. Biotechnol. Biochem.* 71:2338–2342. <http://dx.doi.org/10.1271/bbb.70154>.
- Torriani, S., G. Zapparoli, and F. Dellaglio. 1999. Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L-delbrueckii* subsp. *lactis*. *Appl. Environ. Microbiol.* 65:4351–4356.
- Tsuchiya, J., R. Barreto, R. Okura, S. Kawakita, E. Fesce, and F. Marotta. 2004. Single-blind follow-up study on the effectiveness of a symbiotic preparation in irritable bowel syndrome. *Chin. J. Dig. Dis.* 5:169–174.
- Turroni, F., M. Ventura, L. F. Butto, S. Duranti, P. W. O'Toole, M. O. C. Motherway, and D. van Sinderen. 2014. Molecular dialogue between the human gut microbiota and the host: A *Lactobacillus* and *Bifidobacterium* perspective. *Cell. Mol. Life Sci.* 71:183–203. <http://dx.doi.org/10.1007/s00018-013-1318-0>.
- Tušar, T., K. Žerdoner, B. Bogovič-Matijašić, D. Pavelšek, E. Benedik, B. Bratanič, N. Fidler, and I. Rogelj. 2014. Cultivable bacteria from milk from Slovenian breastfeeding mothers. *Food Technol. Biotechnol.* 52:242–247.
- Unno, T., J.-H. Choi, H.-G. Hur, M. J. Sadowsky, Y.-T. Ahn, C.-S. Huh, G.-B. Kim, and C.-J. Cha. 2015. Changes in human gut microbiota influenced by probiotic fermented milk ingestion. *J. Dairy Sci.* 98:3568–3576. <http://dx.doi.org/10.3168/jds.2014-8943>.
- van Zanten, G. C., L. Krych, H. Roytio, S. Forssten, S. J. Lahtinen, W. Abu Al-Soud, S. Sorensen, B. Svensson, L. Jespersen, and M. Jakobsen. 2014. Synbiotic *Lactobacillus acidophilus* NCFM and cellobiose does not affect human gut bacterial diversity but increases abundance of lactobacilli, bifidobacteria and branched-chain fatty acids: A randomized, double-blinded cross-over trial. *FEMS Microbiol. Ecol.* 90:225–236. <http://dx.doi.org/10.1111/1574-6941.12397>.
- Veiga, P., N. Pons, A. Agrawal, R. Oozeer, D. Guyonnet, R. Brazeilles, J.-M. Faurie, J. E. T. H. Vlieg, L. A. Houghton, P. J. Whorwell, S. D. Ehrlich, and S. P. Kennedy. 2014. Changes of the human gut microbiome induced by a fermented milk product. *Sci. Rep.* 4:6328 <http://dx.doi.org/10.1038/srep06328>.
- Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73:5261–5267. <http://dx.doi.org/10.1128/AEM.00062-07>.
- Yoon, J. S., W. Sohn, O. Y. Lee, S. P. Lee, K. N. Lee, D. W. Jun, H. L. Lee, B. C. Yoon, H. S. Choi, W. S. Chung, and J. G. Seo. 2014. Effect of multispecies probiotics on irritable bowel syndrome: A randomized, double-blind, placebo-controlled trial. *J. Gastroenterol. Hepatol.* 29:52–59. <http://dx.doi.org/10.1111/jgh.12322>.