Modulation of the fecal microbiota by the intake of a Lactobacillus johnsonii La1-containing product in human volunteers

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Abstract

Lactobacillus johnsonii La1 (La1) is a probiotic strain capable of stimulating the immune system of the host and interfering with gastrointestinal pathogens. This study evaluates how the ingestion of different amounts of La1 influences the main bacterial populations of the fecal microbiota. Eight asymptomatic volunteers participated in the study. After a basal period, they ingested daily 100 mL of a product containing 10^8 CFU mL^-1 of La1 during the first week, 200 mL during the second week and 500 mL during the third week. Fecal samples were obtained at the end of each period and subsequently during 7 weeks. Lactobacilli were determined by culture on MRS agar and La1 colonies were confirmed by ERIC-PCR. The main populations of fecal bacteria were identified by FISH and flow cytometry. At baseline, 37.7% of the total fluorescent bacteria were Eubacterium rectale, 18.3% Fusobacterium prausnitzii, 13.2% Bacteroides, 8.6% Atopobium, 2.30% Clostridium histolyticum, 2.05% Bifidobacterium and 0.95% Lactobacillus. Fecal excretion of La1 increased during the intake period and decreased during the post-ingestion period, so that no La1 was observed in the stools of the volunteers seven weeks after the intake product has been finished. La1 intake increased the populations of C. histolyticum (p = 0.049), Lactobacillus (p = 0.056) and Bifidobacterium (p = 0.067), and decreased those of F. prausnitzii (p = 0.005) while it did not affect Bacteroides, E. rectale and Atopobium populations. These bacterial populations returned to their baseline levels during the post-ingestion period. The regular intake of a La1-containing product beneficially affects the homeostasis of the human fecal microbiota, probably contributing to the health-promoting effects of this probiotic.

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1. Introduction

Fermentation processes of food by lactic acid bacteria have been used since antiquity as a way to avoid pathogen contamination and increase food palatability [1]. In the last decades it has been shown that lactic acid bacteria are associated with a wide range of health benefits for the consumer, including immune stimulation [2], defense against pathogens [3], and stabilization of the gastrointestinal barrier function [4]. In consequence, research focused on the development of probiotic strains of lactic acid bacteria and on the demonstration of their health-promoting effects is receiving considerable attention from the scientific and clinical communities [5].
An important mechanism that may explain the health-promoting effects of probiotics is their capacity to modulate the intestinal microbiota and to maintain an equilibrium between the intestinal populations of beneficial and potentially harmful bacteria. Disruption of the homeostasis of the intestinal microbiota is observed in several disorders such as the inflammatory bowel diseases (IBD), allergies and rheumatoid arthritis, and probiotics have been proposed as an alternative approach for the dietary management of these pathologies [6–8]. However, although modulation of intestinal microbiota homeostasis constitutes a basic aspect of the probiotic concept, few studies have evaluated this property in humans. Tannock et al., for example, have shown that the administration of a milk product containing Lactobacillus rhamnosus DR20 to human volunteers resulted in a significant increase of Lactobacillus and Enterococcus species in feces, without affecting other bacterial populations such as Bifidobacterium, Bacteroides, Clostridium, Escherichia coli or Atopobium [9].

Lactobacillus johnsonii La1 (La1) is another well-described strain currently used for the elaboration of probiotic-containing products. Interestingly, the La1 genome has been recently published and its analysis indicates that it is well adapted to survive and compete in the human small intestine, where it is living in close symbiosis with the host [10]. La1 has been shown to stimulate the local and systemic immune systems in humans [2,11,12] and various studies indicate that it may also exert anti-bacterial activities against gastrointestinal pathogens [3,13,14]. For example, we have recently shown that regular ingestion of a dietary product containing La1 may interfere with H. pylori colonization in children [15], and that more frequent ingestion of the product throughout the day resulted in a more intense effect against this pathogen [16]. In the case of La1, it is not known whether this probiotic strain affects the commensal bacterial flora of human hosts.

In consequence, the aim of the present study was to evaluate in human volunteers how the administration of different amounts of a product containing La1 may affect the composition of the dominant fecal bacterial populations: Bacteroides, Fusobacterium, Eubacterium rectale, Clostridium, Atopobium and Lactobacillus, by using fluorescent in situ hybridization (FISH) and flow cytometry.

2. Subjects and methods

2.1. Subject recruitment

Eight asymptomatic volunteers, 4 men and 4 women (27.3 ± 7.6 years; range [19–40 years]) without previously diagnosed gastrointestinal pathologies or history of antibiotic, antacid, laxative or prokinetic drug treatments, were recruited for the study. The protocol was approved by the Ethics Committee of INTA, and an informed consent form was signed by all the subjects who agreed to participate in the protocol. Volunteers were asked to avoid consumption of other commercial fermented dairy and probiotic products throughout the duration of the study. The product used was offered as a 100 mL bottle containing 14.7 g of carbohydrate, 0.9 g of protein and 0.03 g of lipids, and providing 63 kcal (Chamyto, Nestlé Chile SA, Santiago, Chile). La1 was present in the product at a concentration of $10^8$ CFU mL$^{-1}$; in addition, a non-probiotic strain of L. helveticus was also present in the product at a concentration of $2 \times 10^7$ CFU mL$^{-1}$, as required for the elaboration of the product.

2.2. Protocol design

After a five-day baseline period, volunteers ingested one bottle (100 mL) of the product containing La1 daily during the first week, two bottles (200 mL) during the second week and five bottles (500 mL) during the third week. During the last period of La1 ingestion, simultaneously with the intake of every bottle of the product, volunteers had to ingest, 100 mL of a suspension of $7 \times 10^7$ CFU mL$^{-1}$ Bacillus stearothermophilus spores (Merck, Darmstadt, Germany) as marker of intestinal transit [17]. Fresh fecal samples were obtained on days 5 and 0 (i.e., before treatment), on days 7, 14, 17 and 21 of the treatment period and on days 3, 7, 13, 17, 20 and 49 post-ingestion. Fecal samples were collected in sterile flasks and stored under conditions of anaerobiosis (Anaerocult, Merck, Darmstadt, Germany) at 4 °C until processing in the laboratory, for no more than 4 h. Gastrointestinal symptomatology was recorded throughout the duration of the study.

2.3. Quantification of total Lactobacillus population, La1 and B. stearothermophilus

About one gram of feces was diluted 10-times in sterile PBS and homogenized by mixing with a vortex in presence of six glass beads. Gross particles were pelleted by 2-min centrifugation at 300 g at 4 °C; afterwards, serial dilutions of the suspension were spread on Mann–Rogosa–Sharp (MRS) agar or on Plate Counting Agar (PCA) (Biokar Diagnostics, Beauvais, France) for quantification of total lactobacilli and B. stearothermophilus, respectively. MRS plates were incubated for 48 h at 37 °C under anaerobic conditions, and the PCA plates were incubated for 24 h at 65 °C. Total lactobacilli were quantified by counting colony-forming units on the MRS plates corresponding to the last readable dilution. The colonies suspected to be La1 based on their morphological aspect were grown in MRS and stored in MRS-glycerol at −80 °C until the subsequent
confirmation of their identity through ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus sequences-PCR) [18].

2.4. Lal confirmation by ERIC-PCR

Each stored colony was grown in MRS broth, spread on MRS agar for 48 h at 37 °C and a colony was suspended in 50 μL of Tris–EDTA and heated for 10 min at 95 °C. One microliter of this suspension containing the bacterial DNA was immediately added to a reaction mixture containing 2.5 μL Taq Polymerase (Fermentas AB, Vilnius, Lithuania), 200 μM dNTPs, 3 mM MgCl2 and 1 μM of the primers ERIC-I (5′-ATGTAAGCTCTGCGGATTAC-3′) and ERIC-II (5′-AGTAAGTGTACTGGGGGTGAGCG-3′) in a final volume of 25 μL. Bacterial DNA was amplified by PCR using a M.J. Research (Waltham, MA, USA) thermocycler: the amplification program included an initial step of denaturation (10 min at 95 °C) followed by 40 cycles of denaturation (1 min at 95 °C), annealing (1 min at 48 °C) and extension (5 min at 72 °C), and a final step of extension (7 min at 72 °C). Amplified DNA products were separated by 2% agarose gel electrophoresis, stained with 0.5 ng mL−1 ethidium bromide and visualized on a UV-transilluminator at 254 nm.

2.5. Fecal microbiota analysis by FISH and flow cytometry

FISH analysis was carried out as described by Rigottier-Gois et al. [19]. Eight fecal samples were obtained from each subject corresponding to days 5 and 0 before treatment, days 7, 14 and 21 of the treatment period, and days 7, 13 and 49 post-ingestion. The following probes were used to detect bacterial groups in the fecal samples (Table 1): Eub338 (total bacteria), Bac303 (Bacteroides and Prevotella sp.), Erec482 (Eubacterium rectale–Clostridium coccoides cluster), Lab158 (Lactobacillus and Enterococcus spp.), Ato291 (Atopobium, Eggerthella, and Collinsella spp.), Fprau645 (Fusobacterium prausnitzii cluster), Bif164 (Bifidobacterium spp.), Chis150 (Clostridium histolyticum cluster). Oligonucleotidic probes were 5′-labelled with indodicarbocyanine (Cy5) or fluorescein isothiocyanate (FITC) and purified by HPLC (Integrated DNA Technologies, Coralville, IA, USA). Two-hundred microliters of the fecal sample diluted and homogenized in PBS were fixed overnight in 4% paraformaldehyde and stored at −80 °C until processing. The fixed samples were washed in TE buffer (10 mM Tris–HCl, 1 mM EDTA), and permeabilized with lysozyme (1 mg mL−1 in TE buffer) for 10 min. After centrifugation, the pellet was washed in PBS and resuspended in hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl, pH 8.0, 0.01% SDS, and 30% formamide). 50 μL of this suspension was mixed with 4 μL of the FITC-labeled EUB338 probe (50 ng μL−1) and 4 μL of another, genus-specific, Cy5-labeled probe as described in Table 1. After a 16 h incubation at 35 °C in the dark, the bacteria were washed for 20 min at 37 °C with 200 μL of washing buffer (0.065 M NaCl, 20 mM Tris–HCl, pH 8.0, 5 mM EDTA, pH 8.0, and 0.01% SDS) and resuspended in 200 μL of sterile PBS. One mL of sterile PBS was added to 50 μL of the hybridized samples; 2 μL of a solution of microsphere standard beads 6 μm in diameter (Bacteria Counting Kit, Molecular Probes, Eugene, USA) at known concentrations and according to the manufacturer’s instructions were added in the tube containing the Lac158 probe. These served as an internal standard to calibrate sample volumes and to determine absolute counts of Lactobacillus/Enterococcus, for comparison with MRS counts. Samples were analyzed by flow cytometry, using a FacsCalibur flow cytometer (Becton–Dickinson, Franklin Lakes, USA). An air-cooled argon ion laser (488 nm) and a red diode laser (635 nm) were used for excitation, and the green and red signals of the bacteria and beads were collected in the FL1 (515–545 nm) and FL4 (653–699 nm) detectors, respectively. The acquisition threshold was set on forward scatter signals (FSC) and 100,000 fluorescent events were stored in list mode files. Subsequent analysis was performed by using WinMDI software (version 2.8; http://facs.scripps.edu/software.html). Cell numbers corresponding

### Table 1

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<tr>
<th>Oligonucleotidic probes used in the FISH study</th>
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<th>OPD code</th>
<th>Refs.</th>
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to every bacterial group were determined by combining in one hybridization tube the EUB 338-FITC probe with one the group-specific probe labeled with Cy5. An FL1 histogram was built to determine the total number of bacteria present in the sample and hybridizing with EUB 338. A gate was created in the FL1 histogram and a FL4 histogram was subsequently used to determine the proportion of cells hybridizing with the group-specific Cy5-probe. This proportion was corrected by eliminating the background of red fluorescence, determined by using the NON EUB 338-Cy5 probe as a negative control. Results were expressed as the proportion of cells hybridizing with the specific-group-Cy5 probe in relation to the total bacteria hybridizing with the EUB 338-FITC probe.

2.6. Statistical analysis

Concentrations of total Lactobacillus and La1 were expressed as log_{10} (CFU or cells) per gram of stool (means ± SEM). Statistical analysis was carried out by using Statistica package software (StatSoft, Tulsa, OK, USA). FISH results were expressed as the percentage of the total fluorescent events labeled with the Eub338 probe (means ± SEM). Changes in the percentages of the bacterial populations were analyzed by Friedman analysis of variances for repeated measurements after angular transformation and, in case of significant results, differences were analyzed by the Wilcoxon test.

3. Results

3.1. Subjects’ response

All the volunteers completed the study protocol and showed good tolerance for the product, reporting only mild increases of borborygmi during the third week when they ingested 500 mL per day of it.

3.2. Fecal determination of total Lactobacillus population and La1

High counts of Lactobacillus were observed in all the volunteers. As shown in Fig. 1, about 8.44 ± 0.33 log CFU g^{-1} of stool were observed in the basal period; these high concentrations were only slightly affected by La1 intake (Friedman ANOVA: \chi^2 = 18.4; p = 0.049), with lower levels of Lactobacillus observed at days 13, 17 and 20 post-ingestion, compared with the levels seen during the first week of La1 intake.

Quantification by FISH and flow cytometry of total lactobacilli with the Lab158 probe specific for Lactobacillus/Enterococcus species, was carried out with the use of glass beads as described. Basal levels (about 8.0 log cell g^{-1}) were similar to those previously observed by culture in MRS agar, and no changes were observed during ingestion and post-ingestion periods (Friedman ANOVA: \chi^2 = 4.50; p = 0.72) (data not shown).

Fecal concentrations of La1 were evaluated by ERIC-PCR, a recently described typing technique first used with enterobacteria. The profiles generated by PCR have been shown to be resolutive for strains within a single species and this method allowed identification of La1 within the L. johnsonii species [18]. In Fig. 2, the profiles by ERIC-PCR of isolated colonies of fecal lactobacilli grown in MRS agar are compared with a positive control strain of La1. No La1 was found in any volunteer at baseline and the intake of this probiotic during the ingestion period resulted in its appearance...
in the stools of all the subjects, as shown by ERIC-PCR (Fig. 2). Fecal concentrations of La1 increased with the amount of product ingested, resulting in concentrations exceeding $10^6 \text{ g}^{-1}$ at the end of the ingestion period (Friedman ANOVA: $\chi^2 = 25.8; p = 0.0025$). At this point, however, La1 represented only the 0.63% of the total lactobacilli. Once the intake of the product with La1 ended, the fecal excretion of the probiotic regularly decreased, following the same decline pattern as that of the $B.\ stearothermophilus$ spores, which are passively cleared by the intestinal peristalsism (Fig. 1). Fecal excretion of La1 was observed in 7/8 volunteers on day 7 post-ingestion and in 1/8 volunteers on day 17 post-ingestion. On day 49, i.e., 7 weeks after the end of the ingestion period, no La1 excretion was detected.

3.3. Evaluation of fecal populations by FISH

Fig. 3 shows how the different fecal bacterial populations were affected by the ingestion of the La1-containing product. The three main populations observed at baseline were $E.\ rectale$, $F.\ prausnitzii$ and $Bacteroides$ which represented 37.7%, 18.3% and 13.2% of the total bacteria detected, respectively (Fig. 3(a)). The $Atopobium$ cluster represented 8.6% while $Bifidobacterium$, $Lactobacillus$ and the $C.\ histolyticum$ cluster were present as subdominant populations representing only 2.05%, 0.95% and 2.30%, respectively, of the EUB338-positive bacteria (Fig. 3(b)). All these populations represented 80% of the total bacteria detected by the universal EUB338 probe.
La1 ingestion resulted in the alteration of some of the bacterial populations evaluated in this study. A significant decrease was observed in the F. prausnitzii cluster (Friedman ANOVA: \( \chi^2 = 20.1, p = 0.005 \)) at days 21 of the period of ingestion and at day 7 post-ingestion, compared with baseline (14.9 ± 2.4%, \( p = 0.049 \) and 12.7 ± 1.8%, \( p = 0.036 \), respectively) afterwards the levels of this population returned to baseline. No changes were observed for the Bacteroides and E. rectale populations (Friedman ANOVA: \( \chi^2 = 8.17, p = 0.32 \) and \( \chi^2 = 7.08, p = 0.42 \), respectively).

As shown in Fig. 3(b), La1 intake increased the proportion of the C. histolyticum cluster (Friedman ANOVA: \( \chi^2 = 14.2, p = 0.049 \)), as well as the Bifidobacterium and the Lactobacillus/Enterococcus populations (Friedman ANOVA: \( \chi^2 = 13.2, p = 0.067 \) and \( \chi^2 = 13.7, p = 0.056 \), respectively), while no changes were observed in the Atopobium cluster (Friedman ANOVA: \( \chi^2 = 7.08, p = 0.42 \)).

4. Discussion

Modulation of the intestinal microbiota is basic in the concept of probiotic utilization. Although La1 is a probiotic whose health-promoting effects are well described, data about its effect on human intestinal microbiota are scarce. Access to colonic lumen requires invasive methods and in consequence, most of the studies evaluating the effect of dietary manipulations of the microbiota have to be carried out on fecal samples; however it is important to take into account that the fecal microbiota is not an exact reflection of the cecal microbiota [28]. The results of the present study show that the regular ingestion of a La1-containing product affects the fecal bacterial populations of healthy human volunteers. Bifidobacterium, Lactobacillus and C. histolyticum cluster populations were increased as percentages of the total bacteria detected by the Eub338 probe, while the cluster corresponding to F. prausnitzii was decreased and those of Bacteroides, Atopobium and E. rectale were not affected by La1 ingestion. Such an effect is probably related to the ability of La1 to survive its transit along the gastrointestinal tract, as shown by the fecal excretion of living La1, and to its presence in the intestinal lumen at concentrations allowing health-promoting effects [2,3,6,15]. However, after completing the period of administration of the product, La1 was rapidly eliminated from the intestinal lumen, following the pattern of B. stearothermophilus spores clearance and the affected bacterial populations generally tended to return to their baseline levels. These findings confirm that a probiotic such as La1, although of human origin, acts in most people as an allochthonous microorganism which transits along the gastrointestinal tract of the host but without colonizing it. This is due to the efficient barrier function exerted, among others, by the autochthonous microbiota. Compared with other human studies [9,29], high concentrations of total lactobacilli (>10^8 g⁻¹ of stool) were detected in our subjects by culture methods as well as by FISH and flow cytometry. It is possible that this relatively important population of autochthonous lactobacilli acts as a strong competitor for La1 and that this may explain why La1 levels were not higher than 10^7 g⁻¹ of stool, in spite of the daily consumption of 5 × 10^10 La1.

The increase of C. histolyticum cluster, Bifidobacterium and Lactobacillus is probably favored by the decrease of the dominant populations of F. prausnitzii and perhaps other species not detected by the probes used in this study. The increase in Bifidobacterium population during the ingestion and post-ingestion periods is interesting and confirms recent observations we carried out in infants who received a La1-supplemented milk-formula for 7 weeks (Bruner et al., submitted for publication). In these infants, La1 intake increased the Bifidobacterium population from less than 5% of the total bacteria at baseline to a level of about 20% after 7 weeks; however this was not associated with changes in Clostridium, Bacteroides or Enterococcus populations. In relation with these findings, it is interesting to note that the recent description of the La1 genome [10] indicates that this microorganism possesses the metabolic pathways necessary to synthesize and release fructan molecules in the gut lumen. Such molecules are known to act as prebiotics [29], selectively stimulating the growth of endogenous Bifidobacterium populations in the colon, as it was observed in this study. It is probable that this “prebiotic” property is not general to all Lactobacillus sp. but is specific of La1 as the regular ingestion of L. rhamnosus DR20, for example, was not associated with changes in fecal bifidobacteria concentrations in humans [9]; these authors did not observe alterations of the populations of Bacteroides, Clostridium, Eubacterium or Atopobium associated with DR20 intake. In opposition with these findings, we observed that La1 ingestion resulted in a lower F. prausnitzii population. Regulation of Fusobacterium levels may be interesting for the host’s health as Fusobacterium species isolated from patients with ulcerative colitis have been shown to be capable of inducing colitis in rats, implicating these microorganisms as a possible etiologic factor in IBD [30]. Eubacterium has also been implicated in IBD [31] but in this study we do not observe any significant effects of La1 on the E. rectale cluster population, perhaps due to the high variability in their basal levels. When our results are compared with those of Tannock et al. [9], it is interesting to notice that La1 and DR20 exert different effects on the human fecal microbiota. This suggests that every probiotic strain possesses specific properties or activities that influence their interrelationships with the autochthonous bacterial populations in the human gut.
ecosystem. More studies are necessary to clarify this point.

In conclusion, our results show that consumption of a La1-containing product by human volunteers modulates their fecal microbiota, as shown by the higher levels of Lactobacillus, Bifidobacterium and C. histolyticum populations and lower levels of F. prausnitzii. They also confirm that La1 does not colonize permanently the gastrointestinal tract of the host, but that it is rapidly cleared when its intake is stopped. This results in the return of the affected bacterial populations to their baseline levels. These observations suggest that regulation of intestinal homeostasis may be an important factor in the health-promoting effects attributed to La1.

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