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Effects of the consumption of *Bifidobacterium lactis* HN019 (DR10™) and galacto-oligosaccharides on the microflora of the gastrointestinal tract in human subjects

Pramod K. Gopal[a,*], Jaya Prasad[a], Harsharnjit S. Gill[b]

[a]*Bioscience Section, Fonterra Research Centre, Private Bag 11029, Dairy Farm Road, Palmerston North, New Zealand*

[b]*Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand*

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Abstract

The effects of dietary consumption of milk derived oligosaccharides and a probiotic bacterium *Bifidobacterium lactis* HN019 (also known as DR10™) on the microbial composition of the gastrointestinal tract of human subjects was examined using a randomized, double blind, placebo-controlled study design. Thirty subjects (age range 20–60 years) were divided randomly into three groups. After two weeks of a pre-intervention period, volunteers in group one consumed a reconstituted milk containing 2.4 g of galacto-oligosaccharides per day, volunteers in group two consumed reconstituted milk containing 3×10^{10} CFU of *B. lactis* HN019 per day, and group three volunteers consumed reconstituted milk without galacto-oligosaccharides or probiotic bacteria. The feeding period continued for four weeks followed by a wash out period of two weeks. Faecal samples were collected at weekly intervals and analysed for eight major groups of microbes associated with human gastrointestinal tract. Subjects receiving reconstituted milk containing either galacto-oligosaccharides or probiotic bacteria (*B. lactis* HN019) exhibited a significant increase in the faecal counts of both lactobacilli ($p < 0.004$) and bifidobacteria ($p < 0.0002$). In comparison, little or no changes were observed in lactobacilli and bifidobacteria counts in subjects who consumed reconstituted milk without supplementation with either galacto-oligosaccharides or probiotic *B. lactis* HN019 (placebo control group). Furthermore, *B. lactis* HN019 survived the digestion through human gastrointestinal tract and colonised transiently. It is believed that desirable bacteria including members of the genera bifidobacterium and lactobacillus, have a positive impact on human health. The results presented in this study demonstrate that dietary supplementation with *B. lactis* HN019 or galacto-oligosaccharides

* Corresponding author. Tel.: +64-6-350-4600 ext 7062; fax: +64-6-350-4658.
E-mail address: pramod.gopal@fonterraresearch.com (P.K. Gopal).

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increases the proportion of bifidobacterium and lactobacillus in the human gastrointestinal tract and hence may improve human health.

Keywords: Bifidobacterium lactis; DR10$^{TM;}$ Faecal microflora; Galacto-oligosaccharides; Gastrointestinal tract; Lactobacilli; Prebiotics; Probiotics

1. Introduction

The intestinal microflora of healthy humans is mainly confined to the large intestine. At least forty different species of bacteria are commonly detected in the colon and many more have been occasionally reported from the microbiological analysis of human faeces [1,2]. Some of these species are present in large numbers with population sizes of up to10^{10} CFU/g of faeces. In general terms, intestinal bacteria may be divided into species that are potentially harmful, benign, or beneficial towards the host [2,3]. In healthy individuals, there is a natural balance between these types of bacteria. Beneficial bacteria, which include species from the genera lactobacillus, bifidobacterium, and occasionally streptococcus, enteroccus, and bacteroides, play useful roles in aspects of nutrition and prevention of disease [2]. Health promoting effects include production of essential nutrients such as vitamins and organic acids, systemic effects on blood lipids, prevention of gastric infections and stimulation of immune function [2]. Furthermore, the protective effects of beneficial colonic bacteria also include detoxification, and binding of carcinogens [4]. However, undesirable intestinal bacteria including species from the genera clostridium, veillonella, staphyloccocus, proteus, and sometimes enterococcus, escherichia, and bacteroides, produce substances that are potentially harmful to the host, such as putrefactive products, toxins and carcinogens [2]. These substances may not have an immediate detrimental effect on the host but they are thought to be contributing factors in promotion of cancers, hypertension and arteriosclerosis [2,3].

Among the major genera of colonic bacteria, bifidobacterium and lactobacillus are believed to have positive effects on the human host [5,6]. The beneficial effects of these genera are believed to be a result of their metabolic activity, their ability to physically interfere with the adhesion of pathogenic species to surfaces of intestinal cells and their ability to enhance the host immune function. Examples of the beneficial effects due to metabolic activity include production of short chain fatty acids and vitamins, enhanced digestion and absorption of nutrients and removal of toxic and other undesirable metabolites. An increase in the number and activity of bifidobacteria and lactobacilli in the colon is therefore likely to be desirable. One approach to achieve this effect has been oral administration of live, beneficial microbes also known as “the probiotic approach”. The rationale of this approach is to facilitate delivery of a viable strain of selected bacteria to the colon where it becomes metabolically active and capable of producing beneficial health effects for the host.

A second strategy is to increase the indigenous population of beneficial bacteria by providing a selective carbon and energy source that provides them with a competitive advantage over other bacteria in the ecosystem. This approach uses specific dietary components termed as prebiotics to selectively modify the composition of resident microflora of the

colon [6,7]. Galacto-oligosaccharides are one such group of food components that are known to promote the growth of bifidobacteria *in vivo* [8,9]. Galacto-oligosaccharides are usually derived from the milk sugar lactose by an enzymatic reaction using β-galactosidase (lactase). This group of oligosaccharides can produce unpleasant side effects, such as, flatulence and stomach cramps in lactose intolerant people who generally lack β-galactosidase in their gastrointestinal tract. To ensure that in this study the results were not biased due to the physiology of the subjects no lactose intolerant subjects were included.

Recent studies in our laboratory demonstrated that the dietary consumption of *B. lactis* HN019 enhanced natural immunity in healthy elderly subjects [11–13]. Reports from several laboratories have indicated that a typical daily dose of probiotic lactic acid bacteria needed to confer physiological effects is in the range of 1×10^{10} CFU to 1×10^{11} CFU [14,15] and doses $<1 \times 10^{9}$ CFU/day are ineffective [16]. Our studies, however, have shown that *B. lactis* HN019 is effective in improving the cellular immunity of human subjects at comparatively low daily dose of 5×10^{9} CFU [13]. In the present study we have used both approaches to study the impact of the consumption of a reconstituted milk containing either galacto-oligosaccharides, or a probiotic bacterium *B. lactis* HN019 [10] on the microflora of healthy human subjects.

2. Materials and methods

2.1. Subjects

Thirty healthy volunteers (12 women and 18 men) ranging in age from 20 to 60 years, were recruited for this study. Volunteers were free from any gastrointestinal disease, and did not suffer from lactose intolerance. Ethical approval for this study was obtained from the Massey University Ethics Committee, Palmerston North, New Zealand. All participants in the study gave their written, informed consent. One volunteer withdrew from the study citing an upset stomach as the reason for pulling out. As the study was double blind, at the time of dropping out the identity of milk consumed by this participant was not known. At the end of the study, it was noted that the individual was in the placebo group consuming base milk powder only. Data from this individual were not included in the final analyses.

2.2. Dietary intervention

The dietary intervention used in this study consisted of feeding three types of milk powders. The base milk powder (BP) was a low fat (12% w/w) milk powder. The second milk powder (BP*Oligo*) consisted of the base powder which had been enzymatically treated with β-galactosidase from *K. lactis* (Novo) to produce galacto-oligosaccharides *in situ.* The concentration of galacto-oligosaccharides in the enzymatically modified milk powder (BP*Oligo*) was 8% w/w. Probiotic bacterium *Bifidobacterium lactis* HN019 (DR10^{TM}) was supplied by the Starter Production Unit of Fonterra Research Centre, and blended with the base milk powder to obtain a final probiotic concentration of 10^{9} CFU per gram of milk powder (BP*BL*) .

2.3. Experimental design

The study was designed to be randomised, double-blind and placebo-controlled. Thirty volunteers were randomly divided into three groups of 10 people. Each group consumed one serving per day of 250 mL of reconstituted milk made from either BP, BP*oligo* or BP*BL* (12.0 g/100 mL). The milk powders were supplied to the volunteers in pneumatically sealed sachets containing 30 g of powder. Sachets were reconstituted by subjects immediately prior to consumption by mixing the powder with 250 mL of drinking water according to instructions. There was no perceptible difference in taste between reconstituted plain or probiotic supplemented milk.

The ten-week trial was divided into three periods: pre-feeding, dietary intervention and wash out periods.

1. The pre-feeding period consisted of two weeks. During this period the subjects consumed their normal diet, including no reconstituted milk.
2. The dietary intervention period lasted for four weeks. During this period the three groups of subjects each consumed the three different reconstituted milk powders. They consumed one serving (250 mL) of reconstituted milk powder each day in addition to their normal diet.
3. The wash out period of two weeks was the final step in this feeding trial. As with the first period, subjects consumed their normal diet but stopped consumption of the reconstituted milk powder.

The only dietary restriction imposed on the participants during the entire duration of this study was the prohibition of the consumption of any products with live bacteria (*eg* yoghurt, cultured dairy foods, or dietary supplements).

Faecal samples were collected from all subjects on days −14, −7, 0, 7, 14, 21, 28, 35 and 42. Instructions were given to all participants on how to collect faecal samples in the sterile specimen containers provided. In total each participant provided 9 faecal samples. Samples were processed immediately upon receipt in the laboratory. A portion of the faecal sample was saved and stored at −80°C.

2.4. Bacteriological analysis of faecal samples

All faecal specimens were analysed within 1 h of being received in the laboratory. Faecal suspensions (1 g in 10 mL) were made in Bryant and Burkey's anaerobic diluent [17] using glass beads (3 mm diameter). Further dilutions were made in the same solution anaerobically. One-hundred μL volumes of each dilution were spread on the surface of plates of various media and incubated anaerobically using Anaero-Bag and Pouch-Anaero anaerobic gas generating system (Mitsubishi Gas Chemical Company, Inc., Australia) at 37°C. To enumerate total anaerobes, pre-reduced brain heart infusion broth supplemented with 0.5% (w/w) yeast extract, 5% (w/v) sheep blood, vitamin K_1 (0.1 μg/mL), hemin (5 μg/mL) and L-cysteine hydrochloride (0.05% w/v) was used. For enumerating bacteroides, the above medium, supplemented with the antibiotics kanamycin (75 μg/mL) and vancomycin (7.5 μg/mL), was employed [18]. Bifidobacteria were enumerated using Beeren's medium [19].

Lactobacilli were enumerated on Rogosa agar [20]. For the enumeration of clostridia, the appropriate dilutions were heat treated at 80°C/10 min (to select for spores) and inoculated into pre-reduced Reinforced Clostridial Medium agar (RCM agar; [21]). The clostridium group of bacteria is inherently robust and heat treatment (80°C/10 min) is used to kill all vegetative cells and allows the spores to predominate which are then selectively enumerated. This technique is most commonly used [40] and ensures a more representative measure of the clostria population in complex sample such as faeces. Enterobacteria and enterococci were enumerated on MacConkey agar and Slanetz and Bartley medium [23]. The plates were incubated aerobically for 18 to 24 h at 37°C.

2.5. Detection of B. lactis HN019 by colony hybridisation using an oligonucleotide probe

After counting on Beeren's medium, the colonies of bifidobacteria were transferred onto a nylon membrane (Hybond-N nylon membrane; Amersham Life Science) by direct colony lifts. Subsequently, the DNA from the cells was extracted, neutralised, washed and fixed to the membrane [24]). The nylon membranes were dried at 80^0 C for 10 min, and DNA was fixed to the membrane by UV cross linking (4 min at 302 nm). The *B. lactis* specific primer 5'-GGGAAACCGTGTCTCCAC-3' and Bifidobacterium genus specific primer. 5'-CGGGT-GCTCCCCACTTTCATG-3' were labelled with [α-^{32}P]ATP using polynucleotide kinase (Boehringer) according to the manufacturer's instructions. Hybridisation of the labelled probes and washing of excess probes were performed by employing Rapid-hyb buffer (Amersham Life Science). After washing the membrane, the filter was subjected to autoradiography [25].

2.6. Oligosaccharides in β-galactosidase treated milk powder

Total carbohydrates were extracted from the enzyme treated base milk powder by precipitating the proteins [26]. Briefly, 2 g milk powder was dissolved in 100 mL of water and incubated at 60°C for 15 min. Proteins were precipitated by adding 1.8% (w/v) barium hydroxide, followed by 2.0% (w/v) zinc sulphate. The precipitated proteins were removed by centrifugation (10, 000 $\times$ g for 10 min). The supernatant was concentrated by rotary evaporation and the amount of total carbohydrates extracted was determined by the phenol-sulphuric acid method [27]. Total carbohydrates were further analysed for the type and relative proportions of different oligosaccharides by Fluorophore Assisted Carbohydrate Electrophoresis (FACE) [28]. Typically, 4 μL of ANTS (8 amino-naphthalene-1,3,6 -trisulphonic acid) was added to 10-20 μg of carbohydrate followed by 4 μL of cyanoborohydride (74 mg/mL in dimethyl sulfoxide). The reaction mixture was incubated at 37°C overnight. After incubation, the reaction mixture was dried in speed-vac and resuspended in an appropriate volume of loading buffer containing 80% (v/v) glycerol. Oligosaccharides were separated on acrylamide gels (32%; w/v). Gels were run at a constant current (15 mA/gel) for 3.5 hrs. Oligosaccharides were viewed under UV light and photographed using Alpha Imager 2000 (Alpha Innotech. Corp. San Leandro CA, USA.)

2.7. Statistical analyses

Values given in all Tables and Figures represent means and standard errors. The influence and interaction of the probiotics and galacto-oligosaccharide supplement on bacterial densities were assessed by analysis of variance (ANOVA, Proc GLM, SAS ver 8, SAS Institute, Cory, NC, USA, 2000). The subject to subject variation in the counts of bifidobacteria was used as error term. The type II error was controlled by a power calculation assuming the subject to subject variation (based on previous study) of bifidobacteria counts and to be confident of detecting a 0.7 log count difference. Dunnett's test was used to test the significance of difference between time periods as compared with the base line.

3. Results

3.1. General observations

Reconstituted milk from all three types of milk powders, namely base powder (BP), base powder with oligosaccharides (BP*Oligo*) and base powder with probiotic bacteria (BP*BL*) were well accepted by the volunteers. At the completion of the trial all participants filled out a questionnaire designed to determine the compliance rate and to provide a record of health related symptomatic observations. The compliance rates of the subjects completing the studies were excellent and none of the participants experienced any adverse symptoms related to the consumption of *B. lactis* HN019, galacto-oligosaccharides or base milk powder.

3.2. Galacto-oligosaccharides in β-galactosidase treated base milkpowder (BPOligo)

Fluorophore Assisted Carbohydrate Electrophoresis analyses of carbohydrate extracts from BP*Oligo* clearly showed the presence of galacto-oligosaccharides (Fig. 1). The carbohydrates detected in this milk powder included, glucose, galactose, two disaccharides other than lactose (but no lactose), three trisaccharides and traces of tetrasaccharides. Quantitatively, trisaccharides were the most abundant galacto-oligosaccharides. In comparison, the carbohydrate fraction from the base powder showed the presence of only one band that corresponded to lactose.

3.3. Impact of the consumption of base milk powder (BP)

The faecal microflora in the group consuming BP remained relatively unchanged (Table 1) and any changes observed were not statistically significant. Analysis of variance data showed no significant difference between the different feeding periods (days of analysis).

3.4. Impact of consumption of base milk powder with galacto-oligosaccharides (BPOligo)

The subjects consuming milk powder with oligosaccharides showed differences in faecal population sizes of bifidobacteria and lactobacilli between the three feeding periods (Table

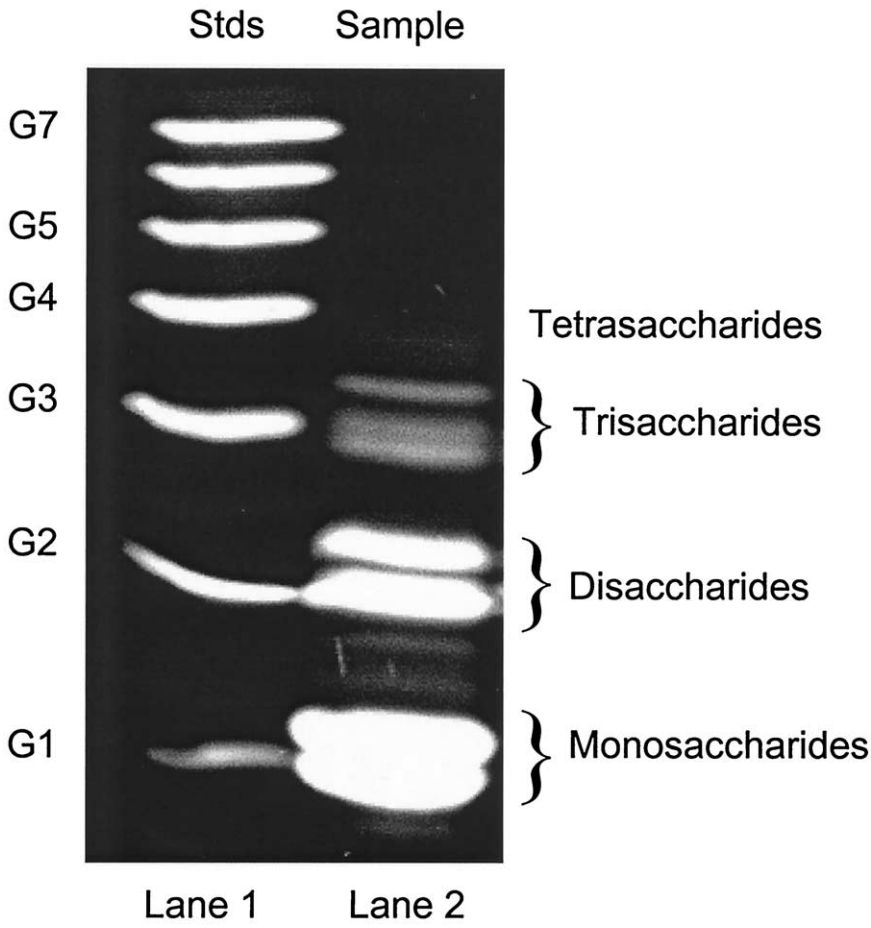

Fig. 1. Galacto-oligosaccharides profile of hydrolysed lactose milk powder (BP*Oligo*). Total carbohydrates were extracted from lactose hydrolysed milk powder and labelled with ANTS (8 amino-naphthalene-1,3,6−trisulphonic acid). Fluorescently labelled samples were separated on 35% acrylamide gel and viewed under UV. Lane 1, laminari-oligosaccharide standards, lane 2 galacto-oligosaccharides.

2). Firstly, the mean value of bifidobacteria increased from 8.67 (at −14 d, pre-dietary intervention period) to 9.40 log units (+28 d, at the end of dietary intervention period). The average increase of 0.73 log units was statistically highly significant ($p < 0.001$). Similarly the lactobacilli population also showed an increase of 0.94 log units at the end of 28 days of dietary intervention, which was again statistically significant ($p < 0.01$).

Furthermore, when the effects of oligosaccharide feeding on lactobacilli populations were analysed at an individual level, some interesting observations were made. Unlike bifidobacteria, the lactobacilli population of individuals showed relatively large variations. Individuals with lower base line lactobacilli counts responded better to dietary intervention than in individuals with higher initial counts. This observation is illustrated in Figure 2. Subjects A and B had lower initial lactobacilli counts *ie* log 7.1 and log 7.5, respectively (at 0 day). At the end of 28 days of dietary intervention lactobacilli count of subject A increased to log 8.57

Table 1
Impact of the consumption of base milk-powder on microflora of the gastrointestinal tract of human subjects[1]

Micro flora	Pre-Study	During-Study				Post-Study
	−14 day	+7 day	+ 14 day	+21 day	+28 day	+49 day
Bifidobacterium	8.66 ± 0.10	8.58 ± 0.16	8.59 ± 0.23	8.44 ± 0.31	8.73 ± 0.08	8.47 ± 0.21
Lactobacilli	7.63 ± 0.32	7.90 ± 0.41	7.91 ± 0.35	7.72 ± 0.043	7.09 ± 0.60	7.45 ± 0.38
Enterobacteria	6.31 ± 0.22	6.77 ± 0.32	6.08 ± 0.47	6.19 ± 0.36	6.27 ± 0.35	6.27 ± 0.50
Faecal streptococci	6.23 ± 0.22	5.93 ± 0.32	5.96 ± 0.30	6.14 ± 0.38	5.96 ± 0.37	5.29 ± 0.33
Clostridia	5.52 ± 0.25	5.21 ± 0.32	5.44 ± 0.29	5.28 ± 0.32	5.56 ± 0.30	5.74 ± 0.15
Bacteroides	9.05 ± 0.08	9.09 ± 0.16	9.04 ± 0.11	8.96 ± 0.11	9.04 ± 0.08	8.94 ± 0.12
Total anaerobes[2]	9.96 ± 0.07	9.95 ± 0.1	10.00 ± 0.08	10.02 ± 0.14	10.14 ± 0.11	10.00 ± 0.08

[1] Data are expressed as mean $\log^{10}$ number per gram wet faeces ± standard error.
[2] Total anerobes constitute strictly (obligately) anaerobic bacteria including *Bacterioides, Eubacterium, Peptostreptococcus, Veillonell etc*.

and subject B to 9.06 (an increase of log 1.47). However, in case of subjects C and D who showed a higher initial lactobacilli count, the increase in lactobacilli population was only log 0.4 and log 0.25, respectively (Figure 2).

Enterobacteria population decreased (by a level of 0.56 log units) and faecal streptococci increased at the end of the 28 days of feeding. These changes in population were statistically non significant.

The counts of both bifidobacteria and lactobacilli decreased to levels typically observed during the pre-feed period once the consumption of milk powder with oligosaccharides discontinued.

Table 2
Impact of the consumption of milk powder with oligosaccharides on the microflora of the gastrointestinal tract of human subjects[1]

Micro flora	Pre-Study	During-Study				Post-Study
	−14 day	+7 day	+ 14 day	+21 day	+28 day	+49 day
Bifidobacterium	8.67 ± 0.12	9.02 ± 0.1	9.26 ± 0.10[a]	9.24 ± 0.14[a]	9.40 ± 0.06[b]	8.84 ± 0.18
Lactobacilli	8.17 ± 0.19	8.39 ± 0.19	8.92 ± 0.20	8.89 ± 0.19	9.11 ± 0.09[c]	8.66 ± 0.10
Enterobacteria	5.91 ± 0.28	5.98 ± 0.44	5.82 ± 0.34	5.39 ± .39	5.34 ± 0.34	5.28 ± 0.49
Faecal streptococci	5.47 ± 0.39	5.62 ± 0.51	5.87 ± 0.41	5.93 ± 0.24	6.04 ± 0.37	5.09 ± 0.28
Clostridia	5.37 ± 0.17	4.90 ± 0.36	5.43 ± 0.16	5.29 ± 0.24	5.24 ± 0.21	5.71 ± 0.23
Bacteroides	8.99 ± 0.10	9.10 ± 0.18	8.67 ± 0.24	8.79 ± 0.17	8.95 ± 0.04	8.95 ± 0.04
Total anaerobes[2]	9.94 ± 0.05	10.04 ± 0.10	10.10 ± 0.05	9.90 ± 0.07	10.6 ± 0.06	9.77 ± 0.06

[1] Data are expressed as mean $\log^{10}$ number per gram wet faeces ± standard error.
[a] p (<0.01).
[b] p (<0.0003).
[c] p (<0.004).
[2] Total anerobes constitute strictly (obligately) anaerobic bacteria including *Bacteroides, Eubacterium, Peptostreptococcus, Veillonella etc*.

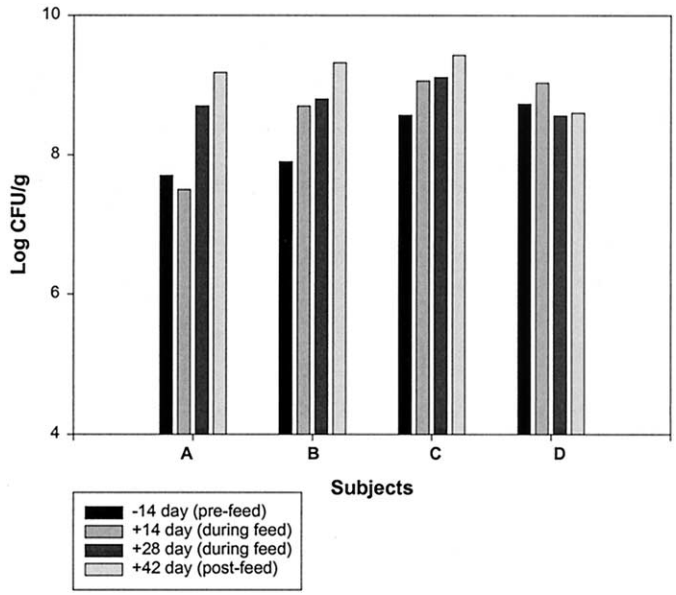

Fig. 2. Impact of the consumption of milk powder with oligosaccharides (BP*Oligo*) on lactobacilli populations of human subjects. Changes in the size of lactobacilli population were monitored in individual human subjects during the dietary intervention with milk containing oligosaccharides. Individuals with lower initial count of lactobacilli (subjects A and B) showed a more significant increase after dietary intervention in comparison with individuals who had higher initial count of lactobacilli (subjects C and D).

3.5. Impact of the consumption of base milk powder with B. lactis HN019 (BPBL)

The group of subjects consuming base milk powder containing *B. lactis* HN019 (BP*BL*) showed similar effects on the population sizes of bifidobacteria and lactobacilli between the three feeding periods as observed with consumption of milk powders containing oligosaccharides (Table 3). The bifidobacteria population showed an average increase of 0.42 log units at the end of 28 days of the feeding period [log 9.2 $\pm$ 0.1 from log 8.78 $\pm$ 0.2 (at pre-feed period)]. Statistically, the change in population was significant ($p < 0.001$). Similarly, lactobacilli numbers showed an increase of 1.33 log units at the end of 28 days dietary intervention which was also a statistically significant ($p < 0.01$) change.

Although the absolute change in the bifidobacteria population due to dietary intervention with probiotic bacteria *B. lactis* HN019 was much smaller (0.42 log units) when compared with the change in counts of lactobacilli (log 1.33), the change in bifidobacterial population was statistically more significant ($p < 0.001$) than the change in the population of lactobacilli ($p < 0.01$). This is due to the fact that bifidobacterial population of subjects was stable and there was little subject to subject variation. The situation with lactobacilli was quite different, this group of bacteria showing large subject to subject variation throughout the study period.

On stopping the consumption of BP*BL*, the counts of both bifidobacteria and lactobacilli dropped to the levels observed prior to dietary intervention. A decrease in enterobacteria was observed during intervention with BP*BL* (log 5.38 $\pm$ 0.3 at pre-feed stage to 4.86 $\pm$ 0.3 after

Table 3
Impact of consumption of milk powder with *B. lactis* HN019 on the microflora of gastrointestinal tract of human subjects[1]

Micro flora	Pre-Study	During-Study				Post-Study
	−14 day	+7 day	+ 14 day	+21 day	+28 day	+49 day
Bifidobacterium	8.78 ± 0.07	8.91 ± 0.15	8.98 ± 0.13	8.94 ± 0.15	9.20 ± 0.10[a]	8.61 ± 0.10
Lactobacilli	7.30 ± 0.30	7.97 ± 0.35	8.46 ± 0.24	8.58 ± 0.19	8.64 ± 0.31[b]	7.71 ± 0.25
Enterobacteria	5.38 ± 0.27	5.92 ± 0.27	5.66 ± 0.29	5.24 ± 0.24	4.86 ± 0.31	5.49 ± 0.47
Faecal streptococci	5.54 ± .27	5.63 ± 0.34	5.73 ± 0.29	6.05 ± 0.29	5.65 ± 0.36	5.06 ± 0.47
Clostridia	5.35 ± 0.17	5.22 ± 0.20	5.09 ± 0.26	5.24 ± 0.22	4.95 ± 0.30	5.20 ± 0.19
Bacteroides	8.88 ± 0.17	8.92 ± 0.13	8.48 ± 0.16	8.65 ± 0.15	8.49 ± 0.11	8.45 ± 0.20
Total anaerobes[2]	9.85 ± 0.07	10.01 ± 0.13	9.96 ± 0.04	9.97 ± 0.05	9.95 ± 0.07	9.83 ± 0.16

[1] Data are expressed as mean $\log^{10}$ number per gram wet faeces ± standard error.
[a] p (<0.0002).
[b] p (<0.01).
[2] Total anerobes constitute strictly (obligately) anaerobic bacteria including *Bacteroides, Eubacterium, Peptostreptococcus, Veillonella etc*.

28 days of feeding). This difference was, however, statistically non-significant. Similar trends were observed for clostridia and bacteroides.

3.6. Effect of the consumption of B. lactis HN019 on resident population of bifidobacteria

To determine if the consumption of *B. lactis* HN019 had impact on the composition of the resident bifidobacteria population of the subjects, colony hybridisation with a oligonucleotide probe specific to strain HN019 was used. This technique tracked the proportion of *B. lactis* HN019 colonies from the total bifidobacterial population of the faecal samples during the feeding and post-feeding periods (Figure 3). Results showed large variations. At one extreme, for example, the highest proportion of *B. lactis* HN019 colonies observed in one subject was 68.8%, while the lowest proportion of *B. lactis* HN019 found in this group was only 0.1%. On average, *B. lactis* HN019 strain accounted for 28% of the total bifidobacteria. As expected, none of the faecal samples obtained from subjects during the pre-feed period showed a positive signal for the presence of *B. lactis* HN019.

4. Discussion

The epithelial lining of the human hind gut is covered with a thick layer of bacteria [29]. Although the biological role of this complex and highly adapted microbial community is not completely understood, it is evident that these bacteria exert considerable influence on the biochemistry and the physiology of host gastrointestinal tract. There is evidence in the literature to suggest that some of the indigenous bacterial species may protect the host from disease [2,30], help digest food, and influence the immune system [31]. These functions are attributed to a group of bacteria that are classified as beneficial bacteria. Two genera that

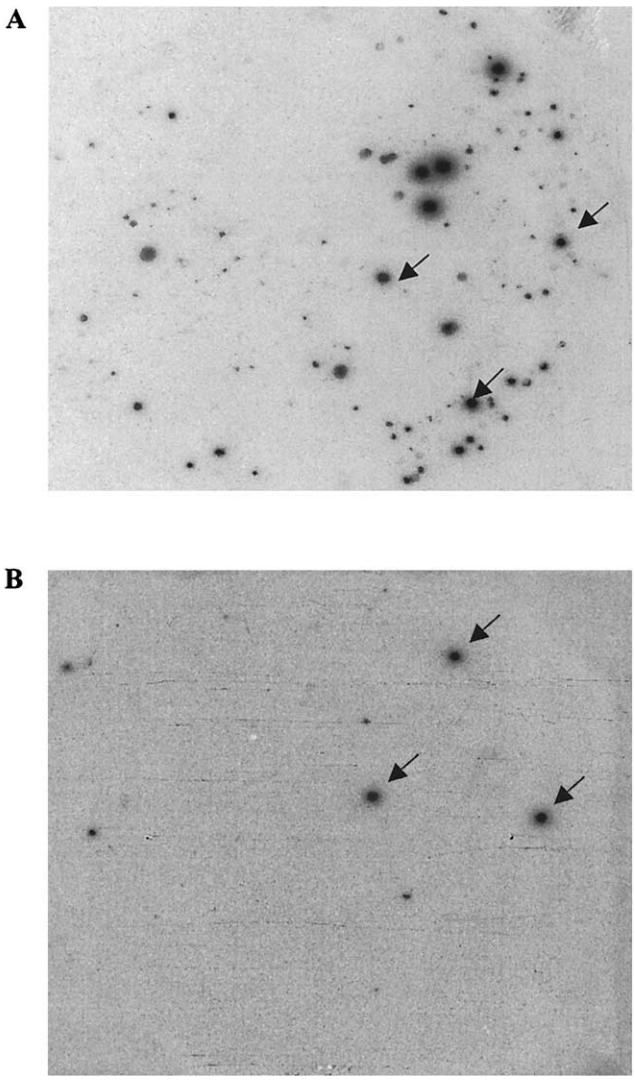

Fig. 3. Tracking *B. lactis* HN019 in faecal samples of human subjects. Faecal samples were analysed for total bifidobacteria count on Beeren's medium. Replica-plates were made by direct colony lifts on nylon membranes. Fixed DNA on membranes were probed with either bifidobacterium genus specific probe (panel A) or with *B. lactis* specific probe (panel B). Panel A shows total bifidobacteria positive colonies, while panel B shows only *B. lactis* positive colonies. Arrows highlight the differences between the two plates.

belong to this group include bifidobacterium and lactobacillus [2]. Results presented in this paper demonstrate that the populations of these two genera can be manipulated in the human gastrointestinal tract through specific dietary interventions.

The subjects in the placebo group (who consumed a standard low fat base milk powder) showed statistically non-significant changes in the gastrointestinal microflora when a comparison between the population sizes of different colonic bacteria was made during feeding and pre- feeding periods of this study. The levels of major groups of bacteria commonly

found in the human gastrointestinal tract were similar to those previously reported [1,32,33]. For example, the total anaerobic flora were in range of $\log^{10}$, bifidobacteria ranged between 8.5 to 9.0 and the enterobacteria up to 6.5.

Consumption of a daily dose of 2.4 g of galacto-oligosaccharides in a milk powder resulted in a statistically significant increase in the population sizes of both the bifidobacteria as well as lactobacilli. Growth promotion of indigenous bifidobacteria by galacto-oligosaccharides has been reported previously [9,34,35]. Our results are also in agreement with the results of Bouhnik *et al*, [8] who tested the effect of prolonged administration of trans galacto-oligosaccharides on bifidobacteria and other colonic flora. The bifidobacterium population showed a significant increase from 8.6 $\pm$ 0.6 to 9.5 $\pm$0.6 log units at end of day 21 ($p < 0.05$) with the consumption of 10 g oligosaccharide/day. The increases in populations of bifidobacteria (8.7 $\pm$ 0.1 to 9.4 $\pm$ 0.06 log units) and lactobacilli (8.2 $\pm$ 0.2 to 9.1 $\pm$ 0.1 log units) observed in the present study are of a similar order. Our results are, however, at variance with the findings of Alles *et al.,* [36] who concluded that daily consumption of trans galacto-oligosaccharides (either 7.5 g/d or 15g/d) did not change the composition of the human intestinal microflora. Similar increases in the population size of bifidobacteria were observed in both galacto-oligosaccharides-fed and control subjects. One of the explanations offered by the authors was the possible adaptation of resident bifidobacteria to the high protein, low fibre background diet used in their study. The results of the present study clearly show that increases observed in the sizes of populations of bifidobacteria and lactobacilli in human subjects were due to the inclusion of 2.4 g of galacto-oligosaccharides in their daily diet. Other studies reported in the literature have linked the increase in counts of bifidobacteria in the large intestine with the consumption of various types of oligosaccharides. Using rats harbouring human faecal flora, Djouzi and Andrieux [37] compared the effect of three oligosaccharides (β-fructo-oligosaccharides, β-galacto-oligosaccharides and α-gluco-oligosaccharides) on the metabolism of intestinal microflora. They reported that β-fructo-oligosaccharides and β-galacto-oligosaccharides were preferred growth substrates for bifidobacteria, which increased in numbers by 2 log units in the faeces of rats when compared with the faeces of rats fed on α-gluco-oligosaccharides or control diets. Similarly, Rowland and Tanaka [38] studied the effects of transgalactosylated oligosaccharides on gut flora metabolism and reported a significant ($p < 0.001$) increase in the total anaerobes, bifidobacteria and lactobacilli with a simultaneous significant ($p < 0.001$) decrease in enterobacteria numbers.

Recently we identified several probiotic strains of lactic acid bacteria [10] and demonstrated their ability to enhance natural and acquired immunity in mice [39]. The immune-enhancing effects of one of these strains, namely, *B. lactis* HN019 were demonstrated in humans [11–13]. These studies also showed that aspects of cellular immunity such as phagocytic capacity of mononuclear and polymorphonuclear phagocytes, and tumoricidal activity of natural killer cells, are enhanced in human subjects as a result of the consumption of *B. lactis* HN019 at a total dose as low as 5×10^9 organisms/day [13]. In the present study we examined the effect of consumption of *B. lactis* HN019 on the microflora of the human gastrointestinal tract. The results showed that consumption of reconstituted milk containing *B. lactis* HN019 at a total daily dose of 3×10^{10} organisms/day increased the number of bifidobacteria and lactobacilli in the colon of human subjects. The change in population sizes

of these groups of bacteria was statistically significant. The dose used in the present study (3×10^{10} CFU of *B. lactis* HN019) is more than 10 fold higher than the minimum dose required to produce measurable effects on the immune parameters in humans [13]. Based on findings of previous research [11–13] and findings presented in this study, it is reasonable to conclude that *B. lactis* HN019, when consumed by human subjects (dose higher than 1×10^{10} CFU per day), are able to have an impact on the gut microflora as well as immune parameters. This conclusion, however, has to be qualified by the fact that results are from different studies and different subjects. As far as the effects of probiotic consumption on the bifidobacterial population are concerned, similar results have been observed in children and adults by other researchers previously. Benno and Mitsuaka [40] reported an increase in the counts of bifidobacteria as well as a remarkable decrease in counts of clostridia in adult human subjects consuming a daily dose of *B. longum.* In a study involving children (ages 15–31 months), Fukushima *et al* [41] reported that feeding of *B. bifidum* strain Bb12 resulted in a tendency of increase in the counts of bifidobacteria and a decrease in the counts of clostridia. Another double-blind randomised study with 20 full-term infants who consumed milk powder containing 10^6 viable *B. bifidum* per gram of milk powder, showed a significant increase in the population of bifidobacteria [42]. A surprising finding of the present study was the observation of a significant increase in population of not only bifidobacteria but also of lactobacilli. One possible explanation for the increase in faecal lactobacilli after consumption of *B. lactis* HN019 could be a decrease in the pH of the colon due to high numbers of bifidobacteria. Being a lactic acid producing bacteria, lactobacilli can grow well at an acidic pH and this may have provided a competitive advantage over other bacterial population groups. Gibson and Roberfroid [2] observed that the mechanism by which bifidobacteria may inhibit the growth of other bacteria probably involves acidification of the environment by virtue of the production of large quantities of carboxylic acids (mainly acetate and lactate) that are the end metabolic product for this genus.

An important finding of the present study is the unequivocal demonstration of the recovery of live *B. lactis* HN019 in the faeces of subjects consuming this strain. In some subjects numbers recovered were as high as 12.5×10^8 CFU/g wet faeces, indicating that the strain was able to reach the colon alive and proliferate. This proves the ability of *B. lactis* HN019 to survive the transit through the human gastrointestinal tract. Interestingly, there was a great variation in the proportion of *B. lactis* HN019 recovered from different subjects (based on plate count) the percentage of bifidobacteria found to be *B.lactis* HN019 ranged from 0.1 to 69% of total bifidobacteria. This variation may be due to differences in the presence of adhesion receptors for lactic acid bacteria amongst individuals, inter-species competition and/or other host factors such as the immune responses to strain used. This finding is consistent with a previous study with *L. rhamnosus* HN001 [22]. In that study, we reported that *L. rhamnosus* HN001 became a dominant lactobacillus strain in the gastrointestinal tract of only a proportion (not all) of the study population during the intervention period. This effect appeared to be related to stability of resident lactobacilli population of the host. Bouhnik *et al.* [8] reported that orally administered *B. longum* reached the intestine but did not proliferate. Kullen *et al.*, [43] demonstrated the survival of an unmodified strain of bifidobacterium in the gastrointestinal tract of human subjects using restriction fragment

length polymorphism of 16S rDNA. They reported a concentration of 67.2 ± 8.5% of ingested bifidobacteria out of the total bifidobacteria.

In summary, the results of this study have demonstrated that the consumption of *B. lactis* HN019 is able to improve the microflora of human gastrointestinal tract by increasing the counts of both bifidobacteria as well as lactobacilli populations; *B. lactis* HN019 not only survives passage through the human gastrointestinal tract, it is also able to proliferate in some individuals. Consumption of milk powders containing galacto-oligosaccharides was also found to influence the human gastrointestinal microecology in a similar way.

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