Effects of a probiotic preparation on the human faecal microflora and biochemical and haematological parameters

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ABSTRACT: The aim of this study was to investigate the effects of supplementation of a probiotic preparation PROBIO-C containing *Lactobacillus acidophilus*, *Bifidobacterium longum*, *L. rhamnosus*, and *Streptococcus thermophilus* on the intestinal microflora of healthy volunteers and the effects on biochemical and haematological measurements. In vitro analyses, which included acid and bile salts tolerance and adhesion tests, were performed first. Probiotic bacteria in PROBIO-C demonstrated adhesion to human intestinal Caco-2 cells. An intervention study was conducted in a single-blind, random, and placebo-controlled trial. Twenty-four healthy volunteers were randomly assigned to one of three treatment groups, one provided with the placebo powder excluding the probiotic, one with 3 g of PROBIO-C with a targeted dose of 1.5×10^{11} CFU of probiotic bacteria, and one with 6 g of PROBIO-C with a targeted dose of 3×10^{11} CFU of probiotic bacteria. The experiment comprised three phases: the control period (week 1) during which no supplementation was given; the test period (weeks 2–4) during which volunteers were given either the placebo sample or PROBIO-C each day; the post-test period (week 5) during which no supplementation was given. The faecal samples were collected once every week throughout the study. The abundances of *Bifidobacterium* and *Lactobacillus* species increased significantly during the test period in both PROBIO-C supplemented groups. In addition, the probiotic supplementation did not result in any significant change in clinical chemistry and haematological parameters. The results from this pilot study indicated that PROBIO-C is well-tolerated and safe.

KEYWORDS: intestinal microflora, intervention study, probiotics

INTRODUCTION

Probiotic, a Greek term meaning 'for life', was initially defined by Lilly and Stillwell in 1954 to refer to any substance secreted by a microorganism that inhibits the growth of another microorganism[1]. Many other definitions were then proposed; and it was not until 1992 that Havennar and Huis In't Veld re-defined the term 'probiotic' as referring microorganisms that can survive in the intestinal tract, modulate the intestinal microflora population, and confer a health benefit to the host[2]. For example, bifidobacteria and lactobacilli are capable of enhancing intestinal resistance to pathogenic infections, reducing cholesterol levels, modulating the immune response, and producing vitamins[3, 4].

Changes in the diet, psychological stress, environmental factors or raging can cause an increase in the number of pathogens. This may further result in dysbiosis of intestinal microflora, which increases susceptibility to infection. Probiotics provide resistance to invasion of pathogenic and opportunistic microorganisms by competitive exclusion and enhancement of host barrier defences[5–7]. Dietary prebiotics are natural or fortified foods and fermented ingredients, which modulate the composition and function of the intestinal microflora, hence conferring a health benefit.

Lactobacilli are facultative anaerobic and catalase-negative organisms[8]. *Lactobacillus* species are capable of colonizing the intestinal flora and provide a niche for vitamin B and vitamin K biosynthesis. *Lactobacillus* species exert anti-tumour activity with regard to colon cancers[9–11], improve clinical symptoms in patients with ulcerative colitis[12], relieve constipation in individuals on low-calorie diets[13], reduce allergy symptoms[14], and lower total cholesterol levels in animal studies[15]. Combined supplementation of *Lactobacillus* and *Bifidobacterium* species may ameliorate symptoms of collagenous

colitis[16]. Heat-killed *Lactobacillus acidophilus* also shortens the recovery time for non-rotavirus diarrhoea in children[17], and prevents antibiotic-associated diarrhoea in hospitalized patients[18]. Allergies may also be prevented by oral supplementation of *Lactobacillus* and *Bifidobacterium* species[19]. *L. rhamnosus* has been shown to have antimicrobial effects against enteric pathogens, such as rotavirus[20–22]. *Streptococcus thermophilus* is commonly found in high- and low-temperature pasteurized milk products, e.g., cheese, and yoghurt. *S. thermophilus* and their secretory products can strengthen the barrier function of the small intestine and stimulate T helper 1 immune responses[23]. *S. thermophilus* ameliorates the clinical symptoms of mucositis in patients receiving chemotherapy[24], and may also decrease the degree of inflammation and increase the thickness of the gastric lining[25].

If microorganisms are to be used as a probiotic, they must be safe, remain viable during both the industrial production processes and intestinal transit, adhere to the intestinal mucosa, and persist in the gastrointestinal tract to exert an effect. Probiotic species must be able to withstand the host's natural defences against ingested microorganisms, and harsh conditions, such as gastric acid and bile salts, which are two major impediments for their survival in the gastrointestinal tract. Bacteria in the two genera of *Lactobacillus* and *Bifidobacterium* species are important residents of intestinal microflora and are known to resist these conditions. It is difficult to predict the survivability of probiotic strains in vivo, and thus the adhesion and resistance to acid and bile salts should be tested in vitro to evaluate their suitability in a multispecies probiotic preparation. Probiotics must have the ability to modulate the composition of microflora. To test the properties needed for useful probiotics, this study first investigated in vitro adhesion and tolerance to acid and bile salts; second, it enumerates important groups by selective agar culturing; and third, it evaluates the effects of the probiotic preparation on routine biochemical and haematological parameters in healthy volunteers in an intervention study.

MATERIALS AND METHODS

Test materials

The PROBIO-C probiotic preparation contains *L. acidophilus* LA107, *Bifidobacterium longum* BL268, *L. rhamnosus* LCR177, and *S. thermophilus* ST136. Other ingredients include xylooligosaccharides, isomalto-oligonucleotides, xylitol, water-soluble dietary fibre, sucralose, and yoghurt flavour powder. The placebo powder had the same recipe as the probiotic preparation, except for the probiotic bacteria, and was identical in appearance and taste.

Acid and bile salts tolerance

One ml of probiotic culture (10^9 CFU) was added to 9 ml of phosphate buffered saline (PBS), which was previously adjusted to pH 2.0, 2.5, or 3.2. PBS with pH 7.2 served as a control. After 3 h incubation at 37 °C, the culture was serially ten-fold diluted and poured onto MRS agar plates. The plates were incubated at 37 °C for 48 h. The colonies were counted and subcultured at pH 2.0 and 37 °C for 3 h[26]. After centrifugation at $4200g$, the pellet was washed once with PBS and resuspended. The bacterial culture was added to 9 ml MRS medium with or without 0.3% ox gall bile salts and incubated for 3, 12, and 24 h. The incubated culture was then ten-fold serially diluted and plated onto MRS agar[27, 28].

Adhesion to human intestinal cells

Seventy to eighty percent confluent Caco-2 cells were initially cultured in a T75 flask. The media was removed and the cells were washed twice with PBS. Then, 1 ml of trypsin/EDTA was added for cell dissociation, and 40 ml DMEM containing FBS and penicillin-streptomycin was used to inactivate the trypsin enzyme. Then 0.5 ml (10^4 cell/ml) of Caco-2 cell suspension was added to each well (24-well plate with coverslips). The plate was incubated with 10% CO_2 and at 37 °C overnight for proper adhesion and growth. On the next day, the cells were washed twice with PBS and refreshed with 0.5 ml DMEM medium without penicillin-streptomycin. Then, 100 µl of bacterial culture (10^7 CFU/ml) was added to each well, and the plate was incubated with 10% CO_2 and at 37 °C for 2 h. At the end of the incubation, each well was washed with PBS three times and then fixed with 200 µl 10% formalin for 30 min. The cells were stained with filtered crystal violet solution in a dark box for 5 min. Excess staining was removed by washing with 0.5 ml of PBS. The coverslip was removed and a drop of PBS was added to prevent drying. The cell count was performed using a Nikon microscope.

Intervention study

The study was approved by the Institutional Review Board of Taipei Medical University (approval number CRC-05-10-05). In total, 24 healthy participants completed the study (8 males and 16 females; average age 24.1 ± 3.5; average weight 55 ± 10 kg),

and these were randomly divided into 3 groups (eight per group): one group received a single-dose of PROBIO-C, the second group received a double-dose of PROBIO-C, and the third group received the placebo. A single dose was 3 g of PROBIO-C powder dissolved in 150–200 ml of water, and doubled amounts of the PROBIO-C powder and water were consumed by the double-dose group.

This study comprised three phases: the control (week 1), test (weeks 2–4), and post-test periods (week 5). During the control period, no PROBIO-C was given. This was followed by a three-week supplementation of PROBIO-C (test period). During the post-test period, the participants stopped taking PROBIO-C. The participants were asked to take the designated dose of PROBIO-C or placebo during weeks 2–4. Faecal samples were collected once every week throughout the study (5 weeks in total) and subjected to enumeration analysis. Blood samples were collected at the baseline and at the end of supplementation period for biochemical and haematological measurements.

Analysis of faecal microflora

Stools were collected in closed containers to maintain anaerobic conditions. The samples were then transferred to an anaerobic glove box. A weighed sample ($\sim$0.5 g) was added to 4.5 ml of anaerobic dilution media (containing five glass beads) and vortexed.

Under anaerobic conditions, the homogenate was ten-fold serially diluted and plated onto MRS agar either by the spread plate or pour plate methods. The plates were cultured at 35–37 °C in an anaerobic glove box or anaerobic chamber, which contained anaerobic packs or with oxygen removal by suction. The resulting colonies were then counted.

Total anaerobic bacterial counts were enumerated by CDC anaerobe blood agar plate. *Lactobacillus* species counts were determined by using MRS Broth (Difco) as the base media with additives of 2-phenylethyl alcohol and bromocresol green. To analyse the total bifidobacteria population, bifidobacteria iodoacetate medium-25 (BIM-25) plates were used. *Clostridium perfringens* population was enumerated using tryptose-sulphite-D-cycloserine (TSC) agar, whereas Endo agar was used for coliform organisms.

Statistical analysis

ANOVA was first used to determine any differences among the experimental phases (control, test, and post-test periods). Tukey's HSD test was then applied to determine any significant differences among groups at the 95% confidence interval.

Table 1 Survival of PROBIO-C probiotics at different pH.

pH	Bacterial counts[†]	
	0 h	3 h later
2.0	10.54 ± 0.15	10.66 ± 0.39
2.5	10.32 ± 0.23	10.51 ± 0.33
3.2	10.63 ± 0.41	10.71 ± 0.21
7.2	10.59 ± 0.54	10.62 ± 0.34

[†] Bacterial counts are converted to $\log_{10}$ CFU/ml.

Table 2 Time course effects of bile salts on the growth of acid-treated (pH 2.0) probiotics.

Time	Bacterial counts[†]	
	MRS	MRS-bile
0 h	10.32 ± 0.28	10.32 ± 0.28
3 h later	10.35 ± 0.23	10.41 ± 0.62
12 h later	10.69 ± 0.36	10.45 ± 0.32
24 h later	10.73 ± 0.85	10.53 ± 0.12

[†] Bacterial counts are converted to $\log_{10}$ CFU/ml.
MRS: MRS only; MRS-bile: MRS with 0.3% bile salts.

RESULTS

This study used an artificial phosphate buffered system to expose lactic acid bacteria to pH 2.0, pH 2.5, pH 3.2, and pH 7.2 for 3 h. The original CFU of PROBIO-C at 10^{10} CFUs/ml could still be maintained after 3 h incubation at pH 2.0 (Table 1). MRS medium with or without 0.3% bile salts was added to the culture which had previously been incubated for 3 h at pH 2.0. After 24 h incubation, the CFU of PROBIO-C cultured in MRS with 0.3% bile salts was not statistically different from that in MRS alone (Table 2). As shown in Table 3, the adhesion of each individual bacterial strain was similar, although the PROBIO-C including four strains demonstrated a higher adhesion (35.7 ± 5.4 CFU/cell) than any of the single strains alone.

Participants received a total of 6×10^{10} CFU bacteria (single-dose group) or 12×10^{10} CFU bacteria (double-dose group). As shown in Table 4, after three-week supplementation the total anaerobic bacterial counts increased from 11.05 ± 0.74 (mean $\log_{10}$ CFU/g wet weight of faeces $\pm$ SD) to 12.1 ± 1.1 $\log_{10}$ CFU/g in participants receiving a single-dose (3 g of PROBIO-C), but returned to the baseline numbers during the post-test period. Similarly, *Lactobacillus* counts increased after 3 weeks

Table 3 Adhesion of PROBIO-C bacteria to the human Caco-2 cells.

Bacteria	Number of adhered bacteria (CFU/cell)
S. thermophilus	25.3 ± 2.6
L. rhamnosus	28.4 ± 5.6
L. acidophilus	23.4 ± 3.9
B. longum	21.9 ± 4.9
PROBIO-C	35.7 ± 5.4

(9.25 ± 0.90–10.78 ± 0.72 $\log_{10}$ CFU/g). There was already a significant increase in the bifidobacteria counts after 2 weeks (10.86 ± 0.65), and the trend continued to the third week (11.10 ± 0.78). However, during the post-test period the bifidobacteria counts returned to the same level as during the pretest period. The TSC and Endo counts for *C. perfringens* and coliform organisms, respectively, did not change significantly during the test or post-test periods. An increase in total anaerobic bacterial count was observed in the group receiving 6 g of PROBIO-C after 2 weeks of PROBIO-C supplementation (10.64 ± 0.91–12.03 ± 0.96), and maintained at 12.0 ± 1.1 at the end of the test period. However, the number returned to the

baseline (11.7 ± 1.1) during the post-test period (Table 4). Lactobacilli counts were higher than the baseline counts after three-week supplementation (8.46 ± 0.91–9.7 ± 1.4), but returned to the baseline during the post-test period. The abundance of bifidobacteria at week 3 of the test period was higher (10.99 ± 0.62) compared to the baseline (9.75 ± 0.56). Abundances of *C. perfringens* and coliforms organisms did not change significantly during the test and post-test periods. For the placebo group, no significant changes were observed in total numbers of anaerobic bacteria, lactobacilli, and bifidobacteria during the test and post-test periods (Table 4). No significant changes were observed in the counts of *C. perfringens* and coliform organisms.

Biochemical and haematological measurements before and after PROBIO-C supplementation are shown in Table 5 and Table 6. No significant difference was observed for clinicobiochemical and haematological parameters. All values were within the normal range of reference values.

DISCUSSION

The ability to adhere to the intestinal epithelium is important for the survival and colonization of probiotics species. Gilliland et al suggested that

Table 4 Average bacterial counts in faecal samples as determined by selective enumeration.

Group[†]	Bacterial counts, mean ± SD ($\log_{10}$ CFU/g wet weight of faeces)				
	week 1[‡]	week 2	week 3	week 4	week 5
Single-dose group (3 g of PROBIO-C)					
CDC	11.05 ± 0.74[a]	10.92 ± 0.72[a]	11.35 ± 0.66[ab]	12.1 ± 1.1[b]	10.98 ± 0.76[a]
MRS	9.25 ± 0.90[ac]	9.6 ± 1.0[a]	10.0 ± 1.0[ab]	10.78 ± 0.72[b]	8.4 ± 1.2[c]
BIM	9.17 ± 0.95[a]	9.83 ± 0.68[a]	10.86 ± 0.65[b]	11.10 ± 0.78[b]	9.24 ± 0.86[a]
TSC	4.4 ± 1.0	4.06 ± 0.64	3.9 ± 1.6	3.6 ± 1.9	3.69 ± 0.88
Endo	5.98 ± 0.50	6.05 ± 0.81	6.31 ± 0.56	6.7 ± 1.4	6.9 ± 1.1
Double-dose group (6 g of PROBIO-C)					
CDC	10.64 ± 0.91[b]	11.48 ± 0.85[ab]	12.03 ± 0.96[a]	12.0 ± 1.1[a]	11.7 ± 1.1[ab]
MRS	8.46 ± 0.91[a]	9.52 ± 0.34[ab]	9.56 ± 0.64[ab]	9.7 ± 1.4[b]	9.38 ± 0.32[ab]
BIM	9.75 ± 0.56[a]	10.50 ± 0.49[ab]	10.7 ± 1.2[ab]	10.99 ± 0.62[b]	10.04 ± 0.61[ab]
TSC	5.4 ± 1.4	4.7 ± 1.5	4.0 ± 1.0	4.2 ± 1.5	3.9 ± 1.6
Endo	6.1 ± 1.2	5.6 ± 1.5	6.66 ± 0.86	6.54 ± 0.88	6.57 ± 0.59
Placebo group					
CDC	11.09 ± 0.68	11.02 ± 0.45	11.33 ± 0.76	12.3 ± 1.1	11.48 ± 0.81
MRS	9.12 ± 0.96	9.08 ± 0.47	8.9 ± 1.2	9.4 ± 1.0	9.00 ± 0.97
BIM	9.43 ± 0.97	9.36 ± 0.54	9.2 ± 1.2	9.5 ± 1.4	9.5 ± 1.0
TSC	4.9 ± 1.2	4.7 ± 1.1	4.6 ± 1.3	4.23 ± 0.67	4.0 ± 1.6
Endo	6.42 ± 0.93	6.2 ± 1.0	6.88 ± 0.61	7.08 ± 0.54	7.14 ± 0.86

[†] CDC: total anaerobic bacterial counts; MRS: *Lactobacillus* species; BIM: *Bifidobacterium* species; TSC: *C. perfringens*; Endo: coliform group.

[‡] Week 1: before the test period; weeks 2–4: during the test period; week 5: during the post-test period.
Values with different superscript letters were significantly different ($p < 0.05$).

Table 5 Effects of PROBIO-C on clinicobiochemical parameters.

Parameter[†]	Baseline	Week 5
TG (mg/dl)	57 ± 19	62 ± 28
Cholesterol (mg/dl)	160 ± 43	166 ± 36
HDL-C (mg/dl)	61 ± 16	62 ± 14
LDL-C (mg/dl)	98 ± 24	101 ± 19
T-CHO/HDL	2.77 ± 0.66	2.88 ± 0.57
AST/SGOT (U/l)	24.7 ± 7.1	25.7 ± 6.8
ALT/SGPT (U/i)	22 ± 10	24 ± 12

[†] TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; T-CHO/HDL: total cholesterol/high-density lipoprotein cholesterol; AST/SGOT: aspartate aminotransferase/serum glutamic-oxaloacetic transaminase; ALT/SGPT: alanine aminotransferase/serum glutamic pyruvic transaminase. Changes between the baseline and at the end of intervention (week 5) are shown. Values are presented as the mean ± SD.

Table 6 Effects of PROBIO-C on haematological parameters.

Parameter[†]	Baseline	Week 5
WBC (10^3/µl)	5.8 ± 1.6	6.0 ± 1.4
RBC (10^6/µl)	4.70 ± 0.90	4.9 ± 1.5
Haemoglobin (g/dl)	13.5 ± 2.6	14.1 ± 1.5
Haematocrit (%)	40.6 ± 7.7	42.2 ± 3.9
MCV (fl)	84 ± 16	86.9 ± 6.0
MCH (pg)	28.0 ± 5.3	28.9 ± 2.4
MCHC (g/dl)	32.2 ± 5.9	33.28 ± 0.98
Plate (10^3/µl)	262 ± 61	266 ± 48

[†] MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: mean corpuscular haemoglobin concentration. Values are presented as the mean ± SD.

bile salts tolerance is critical for lactic acid bacteria survival in the intestine[28]. Interestingly, most probiotics are more tolerant to bile salts than to acids[29]. The results from this study indicated that the 4 strains of bacteria in the probiotic preparation could survive reasonably well at low pH and in the presence of bile salts. For probiotic bacteria to exert their beneficial effects, they must adhere and at least transiently colonize the intestinal flora. In vitro testing of the adhesion ability of probiotic bacteria to human intestinal cells thus provides the first method of screening for suitable species. In this study, the probiotic preparation (4 strains) had better adhesion to human Caco-2 cells than any of the single strains alone. It is possible that other components in the probiotic preparation may enhance bacterial adhesion to the cells.

Mucosal microflora provides a more realistic microbial landscape of an individual. However, invasive procedures, such as biopsies are required to assess this, and certain anatomical regions, including the small intestine, are particularly difficult to sample. Hence faecal microflora have been widely used as a surrogate for the gut microflora. In this study, the consumption of *L. acidophilus* and *B. longum* in the probiotic preparation possibly accounted for increases in total bacterial counts and abundances of *Lactobacillus* and *Bifidobacterium* species. However, such increases were not observed in the post-test period. This phenomenon was also reported in other studies where increased abundances of probiotic species were only observed during the period of consumption. Interestingly, the uptake of the lactic acid bacteria does not necessarily result in their detection in the faecal samples. In one study, *Lactobacillus* counts did not increase after 3 weeks of daily supplementation of 10^9 CFUs of *L. johnsonii* La1[30].

The faecal counts for the probiotic species, such as lactobacilli and bifidobacteria, were elevated during the supplementation period, but once the supplementation ceased the numbers returned to the pretest levels[31, 32]. In this study, the consumption of PROBIO-C including 4 probiotic strains, *L. acidophilus*, *B. longum*, *L. rhamnosus*, and *S. thermophilus*, likely contributed to the increases in the total count of anaerobic bacteria, lactobacilli, and bifidobacteria during the consumption period. Although their numbers then fell to the pretest level, this 'transient colonization' also means that they are safe to consume[32]. A recent study by Kim et al used pyrosequencing to examine the composition of the intestinal microflora in individuals receiving probiotics[33], and the results did not reveal any significant changes. As healthy microflora are fairly robust and not easily altered, short-term supplementation of probiotics may have limited capability to modify the intestinal microflora. On the other hand, individuals with intestinal diseases may benefit from consumption of probiotics, and the effects may be more pronounced than in other individuals. A study on patients with irritable bowel syndrome (IBS) showed similar results, as probiotic supplementation increased the faecal counts of lactobacilli and bifidobacteria, but counts for *Clostridium* species and *Escherichia coli* subgroup remained the same[34]. Despite no change in the abundances of pathogenic groups, patients still experienced significant relief from the IBS symptoms. Various pathogens also

cause intestinal infections, which are characterized by having symptoms of vomiting and diarrhoea. *Lactobacillus* spp. have been shown to display anti-pathogenic properties against *Vibrio cholerae* and *Klebsiella* spp.[35]. Moreover, probiotics, including lactic acid bacteria, have been proposed to alleviate symptoms of coeliac disease[36]. The effects of the probiotic preparation could be further evaluated in individuals with IBS, or inflammatory bowel disease.

Probiotics have a long history of consumption and seem well-tolerated. In fact, some probiotics are often isolated from food[37]. Many probiotic supplements on the market belong to the category of 'functional foods', which means that they are usually not subjected to rigorous safety assessments. Few studies have reported the tolerability and safety of probiotic consumption of one strain and multiple strains. Cox et al demonstrated that probiotic supplementation of a single strain *B. animalis lactis* isolate BI-04 or a combination of *L. acidophilus* NCFM and *B. animalis lactis* BI-07 is generally safe[38]. Except for some minor changes in biochemical and haematological measurements, no significant safety concerns were reported[38]. The authors also stressed the importance of safety assessments on different formulations and doses. The current study found no significant changes in either clinicobiochemical or haematological measurements at the end of consumption compared to the baseline. This study is one of the very few works that concurrently investigated the effects of probiotics on the faecal microflora while also carrying out routine laboratory measurements for safety monitoring.

This study showed that three-week supplementation at either targeted dose 6×10^{10} or 12×10^{10} CFU resulted in detection of *Bifidobacterium* and *Lactobacillus* species in faecal microflora. Routine laboratory measurements also indicated that the probiotic preparation is safe and well tolerated by healthy individuals. The results from this study also warrant further research with a longer supplementation period to examine the long-term effects, and it is anticipated that a reduced abundance of pathogens such as *Clostridium* and coliform groups may also be observed in such works.

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