RESEARCH PAPER

Gut Microbes 3:3, 221-227; May/June 2012; © 2012 Landes Bioscience

Gut Balance, a synbiotic supplement, increases fecal *Lactobacillus paracasei* but has little effect on immunity in healthy physically active individuals

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Keywords: synbiotics, athletes, immune function, prebiotics, acacia gum, Gut Balance, microbiota

Abbreviations: IFNγ, interferon-gamma; IL, interleukin; GI, gastrointestinal; FOS, fructooligosaccharides; GOS, galactooligosaccharides; SCFA, short chain fatty acids; ANOSIM, analysis of similarity; CFU, colony forming units; VO_2 max, maximal oxygen uptake

Synbiotic supplements, which contain multiple functional ingredients, may enhance the immune system more than the use of individual ingredients alone. A double blind active controlled parallel trial over a 21 day exercise training period was conducted to evaluate the effect of Gut Balance™, which contains *Lactobacillus paracasei* subsp *paracasei* (*L. casei* 431®), *Bifidobacterium animalis* ssp *lactis* (BB-12®), *Lactobacillus acidophilus* (LA-5®), *Lactobacillus rhamnosus* (LGG®), two prebiotics (raftiline and raftilose) and bovine whey derived lactoferrin and immunoglobulins with acacia gum on fecal microbiota, short chain fatty acids (SCFA), gut permeability, salivary lactoferrin and serum cytokines. All subjects randomized were included in the analysis. There was a 9-fold (1.2-fold to 64-fold; 95% confidence intervals p = 0.03) greater increase in fecal *L. paracasei* numbers with Gut Balance™ compared with acacia gum supplementation. Gut Balance™ was associated with a 50% (-12% to 72%; p = 0.02) smaller increase in the concentration of serum IL-16 in comparison to acacia gum from pre- to post-study. No substantial effects of either supplement were evident in fecal SCFA concentrations, measures of mucosal immunity or GI permeability. Clinical studies are now required to determine whether Gut Balance™ may exert beneficial GI health effects by increasing the recovery of fecal *L. paracasei*. Both supplements had little effect on immunity. Twenty-two healthy physically active male subjects (mean age = 33.9 ± 6.5 y) were randomly allocated to either daily prebiotic or synbiotic supplementation for 21 day. Saliva, blood, urine and fecal samples were collected pre-, mid- and post-intervention. Participants recorded patterns of physical activity on a self-reported questionnaire.

Introduction

Synbiotic supplements combine probiotics and prebiotics to yield greater efficacy than use of either functional ingredient alone. These supplements exert beneficial health effects by modifying GI microbiota and enhancing immunity. The two most common commercial strains of probiotics are strains from the lactobacilli and bifidobacteria families, while fructooligosaccharides (FOS or inulin) and galactooligosaccharides (GOS) are the prebiotics most often added to foods. A substantial body of animal, in vitro and ex vivo evidence now indicates that these probiotic strains and prebiotics modify GI microbiota and may have immuno-enhancing[1] and health promoting effects[2] although there are conflicting findings in vivo.[3] FOS and GOS may also increase the fecal concentration of SCFA, which may alter immune cell activity by binding to G protein coupled receptors. The growing body of evidence for these functional ingredients supports their use as supplements to enhance health in the general community.

Combining prebiotics and probiotics with other ingredients may augment their effects. Recent technological advances have allowed the manufacture of ingredients derived from plant and animal sources, such as bovine whey derived lactoferrin and immunoglobulins. Lactoferrin and immunoglobulins are present in humoral components of the body and act to prevent and limit infection. Furthermore, they are known to contribute to the biological activity of milk and initial research indicates that bovine whey derived lactoferrin is safe for consumption by humans.[4]

Research with athletes indicates that those more prone to respiratory tract illness have a dysregulated cytokine response to exhaustive exercise.[5] We chose to investigate the

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Submitted: 11/28/11; Revised: 01/24/12; Accepted: 02/02/12
http://dx.doi.org/10.4161/gmic.19579

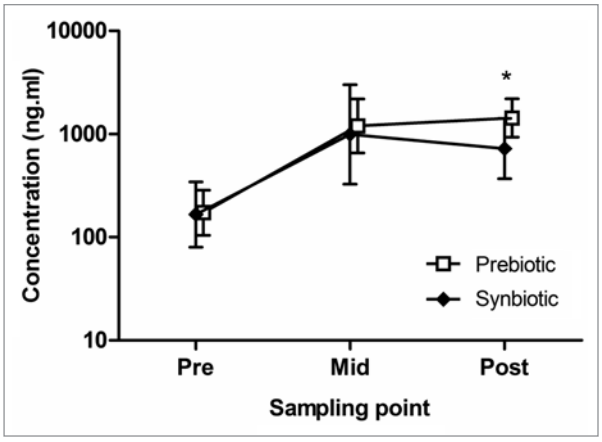

Figure 1. The effect of supplementation on the concentration of IL-16. The values presented are mean and standard deviation of the mean. *p < 0.02.

pro-inflammatory cytokines IL-16, IL-18, IFNγ and IL-12 given their role in regulating $CD4^+$ T cell status, innate immune cell trafficking and the activation of inflammatory mediators.[6,7] Furthermore, altered gut permeability from heavy exercise is proposed to increase mucosal and systemic inflammation via the translocation of bacterial products.[8] Initial studies of probiotics in active individuals have provided insight into the immunomodulatory and health promoting effects of these supplements. Use of this cohort is consistent with the proposition that research in immuno-nutrition studies in healthy people utilize models that challenge homeostasis to determine the ability of the body to respond and adapt to stress.[9]

This study examined the effects of Gut Balance™, containing four probiotics, two prebiotics and bovine whey derived lactoferrin and immunoglobulins with a potential prebiotic (acacia gum) on fecal microbiology, SCFA concentration, cytokines, salivary lactoferrin and gut permeability. Gut Balance™ was chosen as it is available commercially and its constituents have a substantial body of pre-clinical and clinical research completed.[10-12] As the effects of individual probiotic strains and prebiotic additives and their interaction are strain and dose dependent[13] it is necessary to conduct research specific to this formulation. Acacia gum was chosen as a positive control given evidence of its bifidogenic effect and that it is considered a surrogate prebiotic.[14] Employing a positive/active control is consistent with ethical approaches in the use of placebo intervention.[15] New treatments must show greater efficacy than current practices, and a traditional placebo treatment, to justify production and manufacture of a new and novel nutrition supplement.

Results

Subjects. All 22 subjects completed the study. The groups were well-matched on all characteristics (**Table 2**). There were five episodes of mild GI symptoms that included flatulence and stomach rumbles in both groups during supplementation. Both supplements were otherwise well tolerated.

Fecal microbiology and biochemistry. Analysis of similarity of the DGGE patterns indicated that synbiotic supplementation significantly altered the composition of the gut microflora compared with prebiotic supplementation ($R = 0.27$, $p < 0.001$). SIMPER analysis of the DGGE patterns revealed that no one band contributed more than 5% to the dissimilarity between treatments (data not shown). QPCR revealed that there was a relative 9-fold (2-fold to 43-fold; $p = 0.03$) difference in fecal *L. paracasei* between the groups. There were no substantial changes with total Lactobacilli, *L. acidophilus*, *L. rhamnosus*, *B. lactis* and *E. coli* in either group or in the concentrations of the individual short chain fatty acid concentrations (**Table 3**).

Systemic immunity. The concentration of IL-16 over the course of the study is shown in **Figure 1**. Relative to the synbiotic group, there was a 50% (20 to 68%; 90% confidence interval; $p = 0.02$) greater increase in the concentration of IL-16 in the prebiotic group from pre- to post-supplementation. There was no substantial difference between the groups in the resting concentration of IL-18. Covariate analysis did not find any association between changes in microbiota and changes in resting cytokines. The concentration of both IL-16 and IL-18 was characterized by large between- and within-subject variability (~100–300%). No data are reported for IL-12 and IFNγ as the concentration of both cytokines in the samples was below the detection limit of the assay.

Mucosal immunity. There was no substantial effect of supplementation on salivary lactoferrin (-39%; -74 to 41%; 90% confidence interval; $p = 0.3$) or gut permeability (lactulose/mannitol ratio; -75%; -96 to 53%; $p = 0.19$).

Discussion

We show for the first time in healthy physically active individuals that a synbiotic supplement elicits favorable changes in colonic microbiota in comparison to a prebiotic supplement. Supplementation with Gut Balance™ increased the fecal recovery of *L. paracasei* while supplementation with acacia gum, in contrast, was associated with a reduction in fecal *L. paracasei* numbers. There were only trivial effects of supplementation on other species of fecal bacteria analyzed. Both supplements had relatively little effect on the immune system, with the only substantial effect associated with supplementation being a 4-fold increase in the synbiotic group and 8-fold increase in the acacia gum group in resting IL-16 concentration. No substantial effects of supplementation on other cytokines or on parameters of mucosal immunity were evident. An increase in the fecal recovery of *L. paracasei* from supplementation with Gut Balance™ justifies undertaking further research to determine whether supplementation is associated with clinical benefit.

Pre- and pro-biotics purportedly exert their positive effects on the immune system by increasing beneficial species of bacteria colonizing the GI tract. In this study, only the synbiotic supplement fostered a substantial change in fecal microbiota, eliciting a 14-fold increase in the recovery of fecal *L. paracasei.* Given that there were four strains of bacteria in the synbiotic, however, it was expected that a greater number of bacteria would be

recovered following supplementation. Our findings regarding *L. casei* 431® and *B. lactis* BB-12 are in contrast to previous research in which BB-12 was recoverable and *L. casei* 431® was not[16,17] while our inability to recover *L. acidophilus* LA-5 is consistent with the findings of Shioya et al. The lack of recoverable BB-12 following supplementation with the synbiotic is also surprising given the bifidogenic effect reported for FOS and GOS.[18] The results from the present research indicate that the dosage of probiotic bacteria and the dosage of the prebiotics in Gut Balance™ (90 mg Raftiline and 10 mg Raftilose GR per capsule) were not sufficient to elicit further changes in microbiota as evident from the bacterial diversity analysis. That the dosage of prebiotics was too low was further confirmed by the lack of effect of supplementation on fecal SCFA. While this study shows for the first time that the concentration of SCFAs in healthy physically active individuals are similar to the general population, our findings confirm previous research that dosages of 5 to 10 g/day of FOS and GOS are needed to induce changes in fecal bacteria and short chain fatty acid concentrations.[19] The synbiotic formulation may have greater effects on fecal microbiota by removal of FOS and GOS and an increase in the other probiotic species to counts over one billion CFU. Consumption of *L. casei* 431®, in conjunction with *L. acidophilus* LA-5, has been shown to prevent and/or reduce the severity of diarrhea in infants.[20,21] Determining whether increased recovery of fecal *L. paracasei* from *L. casei* 431® supplementation is associated with enhanced intestinal and extra-intestinal health is warranted.

The only effect of supplementation in this study on immunology was an increase in resting IL-16 concentration, which occurred in both treatment groups. However, supplementation with Gut Balance appears to have reduced the magnitude of the increase in IL-16 by 50% relative to acacia gum. The limited effect of Gut Balance™ supplementation on the immune system is similar to a recent study of another synbiotic containing only prebiotics and probiotics, which found that supplementation in healthy adults for six weeks increased the expression of L-selectin but not lymphocyte subsets, phagocytic activity, serum C-reactive protein, ceruloplasmin or other adhesion molecule concentrations.[22] Examination of the training data from participants in the present study indicates that the increase in cytokine concentration was not the result of a change in physical activity patterns from pre- to post-supplementation. While *L. paracasei* can modulate various aspects of innate and adaptive immunity,[23] covariate analysis did not identify any clear trends between supplement-induced changes in microbiota and the increase in IL-16 concentration. IL-16 is a pro-inflammatory cytokine that is chemotactic for immune cells, particularly T-cells.[24] The health benefits of regular exercise are attributed to the promotion of anti-inflammatory effects, in particular by altering cytokine balance.[25] The blunting of IL-16 in this study tentatively indicates that supplementation with Gut Balance™ may augment the anti-inflammatory effects of regular exercise. Given the inherent redundancy within the immune system and the large variance in the range of healthy immune markers, it is necessary to include a wider panel of appropriate markers and utilize systems analysis approaches in future investigations to confirm such an effect.

There was no substantial effect with either Gut Balance™ or acacia gum on gut permeability or the concentration of salivary lactoferrin. While probiotic administration enhances epithelial barrier integrity in vitro and in animal models[26] and in some in vivo studies of critically ill patients,[27] there are few studies reporting effects on healthy individuals. GI permeability was measured in the present study given evidence that individuals undertaking prolonged intense exercise may have higher barrier permeability.[28] Baseline measures of the mean lactulose/mannitol ratio in each group were within normal values and it may be that improvements in GI permeability are more likely to occur only when permeability has been disturbed. Interest in examining the concentration of lactoferrin was based on previous research showing prolonged intense exercise reduces the concentration of lactoferrin by 60%.[29] Future studies examining lactoferrin should focus on cellular activation to antigenic challenge given this may be where benefits are most likely to occur.

The limited effects of supplementation on markers of immunity in this study are consistent with studies of gut health supplements in healthy active populations. Four studies have examined the effects of probiotics on markers of immunity in active populations. While Gleeson and colleagues[30] recently reported reduced respiratory illness and the maintenance of SIgA with four months of *L. casei shirota* supplementation in men and women engaged in endurance exercise, no substantial effects were noted for plasma immunoglobulins, leucocyte subsets or secretion of whole blood culture cytokines. In contrast, a study of *L. fermentum* (PCC) in elite male runners over four months of winter training found that plasma IFNγ was maintained during supplementation but there was no substantial effect on SIgA subclasses or whole blood culture IL-4 or IL-12 secretion.[31] A follow up study of *L. fermentum* (PCC) in 99 well trained cyclists for 11 weeks confirmed the lack of an effect of supplementation on SIgA along with salivary lactoferrin and lysozyme and resting serum cytokines while reporting clinical benefits related to respiratory illness.[32] There were, however, substantial reductions in the acute post-exercise cytokine perturbations in the well trained cyclists following an exercise test to exhaustion. This finding may indicate that probiotics enhance the resilience of the immune system to stress and complements the proposition that health be defined as the ability to adapt to stress.[33] The difference in findings between the studies may relate to the population cohorts (e.g., elite vs. moderately active or sedentary), training mode (e.g., cycling, running) and training load and the strain or dosage of the relevant probiotics.

In conclusion, supplementation with Gut Balance™ in healthy physically active individuals elicited a substantial increase in the recovery of fecal *L. paracasei*. While there were little effects of supplementation on immunity overall, Gut Balance™ also attenuated the increase in resting IL-16 concentration to half that of the those supplementing with acacia gum. Further research focusing on a broader number of cytokines and cellular markers of activity and on conditions associated with aberrant immune responses, particularly inflammatory disorders, should provide further evidence on the usefulness of synbiotics in healthy active adults. In conjunction with evidence that *L. casei* 431® has clinical benefits with regard to infantile diarrhea, the findings from

this study that Gut Balance™ increases the fecal recovery of *L. paracasei* justify undertaking research to determine the clinical effects of this multi-formulation supplement in GI health.

Material and Methods

Study design. The study was a randomized, double blind, parallel controlled trial to compare the effects of a synbiotic supplement with a prebiotic supplement on markers of gut health and immunity. The prebiotic was chosen as the control treatment to determine whether the combination of ingredients in the synbiotic supplement had greater efficacy than the use of a single ingredient. The study consisted of a 14 d pre-intervention period where participants stopped eating yoghurt and supplements that modulate enteric microbiota, a 21 d supplementation period in which subjects consumed with the prebiotic or synbiotic supplement daily and a 14 d post-intervention observation period. Fecal and urine samples were obtained at day 0 (after pre-intervention) and day 21 (end of intervention) for assessment of fecal microbiology and chemistry and GI permeability while serum and saliva samples were collected at days 0, 11 (mid-intervention) and 21 for assessment of immune status. Subjects provided samples for the assessment of fecal microbiology, fecal chemistry and immune status. Subjects were paired on age and maximal oxygen uptake (VO_2 max) and randomly allocated to either prebiotic (acacia gum) or synbiotic supplementation by the study statistician who did not have contact with the subjects. VO_2 max was used as a measure that ensured subjects were undertaking regular endurance activity and performed as previously described in reference 34. During supplementation subjects consumed three capsules of either the synbiotic or prebiotic daily. Subjects were able to consume the supplements with or without food and in the morning or evening. The number of capsules consumed was recorded and all subjects returned their supply bottle following supplementation to verify compliance. The study was conducted according to the guidelines prescribed in the Declaration of Helsinki, and all procedures involving human subjects/patients were approved by the Human Research Ethics Committees of the Australian Institute of Sport and Griffith University. All subjects provided written informed consent.

Subjects. A total of 22 physically active healthy males aged 33.9 ± 6.5 y (mean ± SD) were recruited from the local cycling community and completed the study. Subjects were required to declare their use of dietary and/or ergogenic aids that may influence underlying immune function and/or exercise performance. All subjects on immunomodulatory medications were excluded. Inclusion to the study was dependent upon the subjects not taking antibiotics and supplements or foods with probiotics for 14 d prior to and during the study period. At the start of the study subjects undertook a VO_2 max test as a measure the subject's fitness for inclusion to the study as physically active individuals. A VO_2 max of > 45 $ml/kg^{-1}/min^{-1}$ is indicative of individuals undertaking regular physical activity. The test was performed on an electromagnetic cycle ergometer (Excalibur Sport, Lode NV Groningen).

Product. The synbiotic capsules (Biosource™ Gut Balance, Probiotech Pharma) contained 200 mg Glycomax Immunoglobulin, 50 mg Glycomax Lactoferrin, 100 mg CBAR-Blend-100 (Chr. Hansen A/S, Horsholm, Denmark), which contained 4.6×10^8 *Lactobacillus paracasei* subs *Paracasei* (*L. casei* 431®), 6×10^8 *Bifidobacterium animalis* ssp *lactis* (BB-12®), 4.6×10^8 *Lactobacillus acidophilus* LA-5, 4.6×10^8 *Lactobacillus rhamnosus* GG, 90 mg Raftiline, 10 mg Raftilose GR and 10 mg magnesium stearate. The prebiotic supplement contained 116 mg acacia powder, 23 mg of microcrystalline cellulose, 8 mg of silica colloidal anhydrous British Pharmacopeia/United States Pharmacopeia (BP/USP), 31 mg chocolate flavor, 174 mg calcium hydrogen phosphate, 116.5 lecithin epikuron and 5 mg magnesium stearate BP.

Sample collection. Saliva was collected using an eye spear (Defries Industries Pty. Ltd.). The eye spear was placed between the cheek and teeth for five min, centrifuged for 5 min at 778 g and frozen at -80°C until analysis. Albumin concentration was assessed to control for changes in salivary flow rate. Blood (5 ml) was drawn from an antecubital vein at rest and prior to the VO_2 max test. Blood samples were collected directly into K3EDTA tubes (Greiner Bio-one; Frickenhausen, Germany). Plasma was separated by centrifugation at 4,974 g for 5 min and stored frozen at -80°C until analysis. Subjects were provided with a fecal sample collection kit and a portable -20°C freezer. A fecal sample was collected in a sealable plastic bag and frozen immediately at -20°C. Following collection of the freezers the samples were frozen at -80°C until analysis. Urine samples were collected using a commercial collection kit (ARL Pathology Pty. Ltd.). In brief, subjects were required to eliminate residual urine after an overnight fast and then consume an isomolar solution (120 ml) containing 6.0 g lactulose and 3.0 g mannitol. Participants then collected urine into a sealed flask over a period of 6 h. At the end of this period a 2.5 ml aliquot was taken from the flask and immediately frozen at -20°C. Saliva, serum and urine samples were taken at the same time of the day to control for diurnal variation.

Measures of mucosal immunity. Salivary lactoferrin concentration was measured spectrophotometrically by an enzyme linked immunosorbant assay (ELISA) using a commercial kit (lactoferrin—EMD Chemicals). Albumin concentration was measured by immunoturbidimetric assay on a Hitachi 911 Chemistry Analyzer (Roche). The between-run coefficient of variation was 6.8%. Urinary lactulose and mannitol were analyzed by high-performance liquid chromatography[35] and expressed as the mean of two separate runs.

Measures of systemic immunity. The cytokines analyzed were IL 16, IL-18, IL-12 (p70) and IFNγ. The concentration of plasma cytokines were measured on a Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty. Ltd.). The samples were analyzed on custom manufactured Multiplex Cytokine Kits (Bio-Rad Laboratories Pty. Ltd.) according to the manufacturer's instructions and read using the Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty. Ltd.

Table 1. Quantitative real-time PCR primers.

Target group	Primer name	Primer sequence (5'-3')	Annealing (°C)	Primer conc. (nM)	Reference
Total bacteria	1114f 1275r	CGG CAA CGA GCG CAA CCC CCA TTG TAG CAC GTG TGT AGC C	60	150	45
Lactobacillus group	Lacto-F Lacto-R	AGC AGT AGG GAA TCT TCC ACA CCG CTA CAC ATG GAG	58	500	46 47
Lactobacillus paracasei	LcaseF LcaseR	GCA CCG AGA TTC AAC ATG GGG TTC TTG GAT YTA TGC GGT ATT AG	60	500	48
Lactobacillus rhamnosus	LrhamF LcaseR	TGC TTG CAT CTT GAT TTA ATT TTG GGT TCT TGG ATY TAT GCG GTA TTA G	62	500	48
Bifidobacterium lactis	Bflact2F Bflact5R	GTG GAG ACA CGG TTT CCC CAC ACC ACA CAA TCC AAT AC	60	600	49

Physical activity log and adverse symptoms. Subjects recorded details of their exercise training during the study. For each session, training mileage (km/wk^{-1}), duration (h/wk^{-1}) and intensity (scored on a 1–5 scale: 1, easy; 5, maximal) were recorded. Subjects recorded daily information of symptoms of gastrointestinal illness during the study as previously described to note any adverse or unusual effects or events during supplementation.[36] Symptoms of GI illness included nausea, diarrhea, bloating and pain. A classification of GI illness was made when two or more symptoms were recorded on consecutive days. The severity of symptoms were self-rated as mild, moderate or severe based on the impact of the symptoms on activities for that day, with mild symptoms associated with no change to normal activities, moderate symptoms resulting in a reduction in or modification to activities and severe symptoms requiring the total cessation of normal activities.

Microbial analysis. DNA was extracted according to Abell et al.[37] and quantified using Quant-iT^{TM} Picogreen (Invitrogen). Microbial diversity was examined using a universal bacteria 16S rRNA primer set (907f-1392rgc). The amplified product was analyzed by denaturing gradient gel electrophoresis (DGGE). The PCR and DGGE gel conditions follow the protocol of Abell et al.[37] with the exception that a 35%–70% denaturing gradient was used. Dominant DGGE bands were extracted from the gels and sequenced for putative identification. DGGE banding patterns were analyzed to estimate bacterial diversity for each specimen using GelCompar II version 6.0 (Applied Maths, Inc.) software package and the normalized banding patterns were further analyzed with Primer6 version 6.1.12 and Permanova^{+} addition version 1.02 (PRIMER-E Ltd.).[38] SIMPER analysis was conducted as previously described in reference 39. Total Lactobacilli, *L. paracasei*, *L. acidophilus*, *L. rhamnosus*, *B. lactis* and *E. coli* were quantified by real time PCR. The primer pairs, and their annealing temperature and concentration, used to detect groups and specific bacteria are outlined in **Table 1**.

Short chain fatty acids. Fecal samples were thawed at 4°C, pooled, homogenized and then sub-sampled for analysis. Weighed portions for the determination of free (unesterified) SCFA were diluted 1:3 w/w with deionized water containing 1.68 mM heptanoic acid as an internal standard (Sigma Chemical Co.). Unesterified SCFA were analyzed as described previously in reference 40. A three point linear standard curve containing acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids was used for calibration at concentrations spanning the range of those measured in samples from this study.

Table 2. Differences in key measures between the groups at baseline

Measure	Prebiotic	Synbiotic	Qualitative difference
n	11	11	
Age (y)	31.4 ± 4.9	34.4 ± 3.5	Trivial
Mass (kg)	73.1 ± 4.9	79.1 ± 10.4	Trivial
VO_2 max (mlkg^{-1}min^{-1})	56.4 ± 4.9	57.9 ± 7.3	Trivial
Training load p/week (duration × intensity)	21.3 ± 18.5	21.4 ± 16.8	Trivial

Statistical analysis. The statistical approach to determine the effect of supplementation was based on clinical and statistical significance.[41] Smallest clinical values were derived by standardization; in this case 0.20 of the pooled between-subject standard deviation of the supplement groups. The differences between group means of outcome variables were assessed with a modification[42] of Cohen's scale as described previously in reference 43.

Descriptive statistics of all measures are presented as mean ± standard deviation or mean ×/÷ factor standard deviation. To calculate the factor standard deviation the mean is multiplied by the SD for the upper level while the lower level is calculated by dividing the mean by the SD. DGGE banding patterns were analyzed using the Bray-Curtis similarity matrix as previously described in reference 37. Relationships between diet and DGGE banding patterns were examined using the analysis of similarity (ANOSIM) test (one-way). The R statistic denotes the similarity between two groups with a value of 0 if similarities within diets and between diets are the same on average, and a value of 1 only if all replicates within diets are more similar to each other than any replicates from different diets. All measures were log-transformed before analysis to reduce non-uniformity of error, and permit the effect of the treatment to be analyzed as a percent difference. Differences in the change in mean saliva and serum protein concentrations, and fecal numbers, between the groups were analyzed with a Student's t-test for independent samples (unequal

Table 3. The effect of supplementation on the concentration on fecal variables

Measure	Prebiotic		Synbiotic		Difference in change	
	Mean ×/÷ SD		Mean ×/÷ SD			
	Pre	Post	Pre	Post	Mean (95% CI)	Inference
Total *bacteria**	4×10^8 ×/÷ 4.0	6×10^8 ×/÷ 3.0	6×10^8 ×/÷ 3.0	6×10^8 ×/÷ 3.0	-2% (-65 to 173%)	Unclear
Total Lactobacillus*	5×10^3 ×/÷ 12	2×10^4 ×/÷ 21	10×10^3 ×/÷ 26	1×10^4 ×/÷ 6.0	-37% (-91 to 364%)	Unclear
*L. paracasei**	4×10^2 ×/÷ 4.0	2×10^2 ×/÷ 5.0	2×10^2 ×/÷ 5.0	1×10^3 ×/÷ 5.0	9-fold (25 to 6264%)	Large
L. rhamnosus#	2	8	5	9	-0.1 (-0.5 to 0.3)	Unclear
*B. lactis**	2×10^2 ×/÷ 8.0	5×10^3 ×/÷ 12	3×10^2 ×/÷ 23	3×10^4 ×/÷ 12	4-fold (0.02- to 754-fold)	Unclear
Acetate (μmol/g)	58 ×/÷ 1.3	52 ×/÷ 1.3	54 ×/÷ 1.5	52 ×/÷ 1.4	0.8 (-25 to 35)	Unclear
Propionate (μmol/g)	17.5 ×/÷ 1.4	16 ×/÷ 1.4	16 ×/÷ 1.6	14 ×/÷ 1.4	-9.4 (-32 to 22)	Unclear
Butyrate (μmol/g)	20 ×/÷ 1.3	17 ×/÷ 1.4	18 ×/÷ 1.7	16 ×/÷ 1.7	1.2 (-31 to 49)	Unclear

SD, factor standard deviation; CI, confidence interval; *L. Paracasei*, *Lactobacillus paracasei*; *L. rhamnosus*, *Lactobacillus rhamnosus*; *B. Lactis*, *Bifidobacterium lactis*. *Copies of the 16S rRNA gene per g ww; #samples with recoverable bacteria.

variance).[44] Baseline values of the dependent variable were included as a covariate in these analyses to account for regression to the mean. The extent to which bacterial counts accounted for changes in outcome measures was investigated through covariate analysis. In these analyses the baseline log-transformed bacterial count or the pre-post change in the log-transformed count was the covariate, and the dependent variable was rank-transformed. The effects of supplementation are shown with 95% confidence limits.

Sample size was determined based on variance analysis (standard deviations) from previous studies on the parameters of interest. To demonstrate a difference of 0.20 of the pooled between-subject standard deviation in the salivary immune parameters, which have previously shown the largest variance, a total of nine subjects per group were required to give 80% power at an α level of 0.05. To account for the potential of drop outs 11 subjects were recruited per group.

Disclosure of Potential Conflicts of Interest

A.W.C. receives research support from and provides consultancy services to Probiotec Pharma Pty. Ltd.

Acknowledgments

The study was funded by Griffith University, Probiotec Pharma Pty. Ltd., and the Australian Institute of Sport (AIS) and conducted at the AIS. The authors would like to thank the subjects and members of the Department of Physiology at the AIS for their assistance during the study, particularly Dr Amanda Cox. The authors would also like to thank Corinna Bennett from CSIRO for her assistance in fecal processing and SCFA analysis.

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