

## Research Article

# Consumption of Galactooligosaccharides together with Probiotics Stimulates the *In Vitro* Peripheral Blood Mononuclear Cell Proliferation and IFN $\gamma$ Production in Healthy Men

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Probiotics and prebiotics modify the intestinal environment and could have immunomodulatory effects. The proliferation of spontaneous and phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs) and their production of interleukin-4, interleukin-5, transforming growth factor- $\beta$ 1, and interferon- $\gamma$  (IFN $\gamma$ ) were determined in eighteen men at the baseline and during a 2-week period of probiotics (mixture of *Lactobacillus rhamnosus* GG, *Lactobacillus rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99) and galactooligosaccharides (GOSs) (3.8 g/day). The spontaneous and stimulated proliferation of PBMC increased from the baseline during probiotics+GOS ( $P < 0.001$ ). The secretion of IFN $\gamma$ , but not other cytokines, by stimulated PBMC increased during the same period ( $P < 0.05$ ). In conclusion, the consumption of this probiotic mixture including GOS appears to increase the capacity of PBMC to proliferate and release IFN $\gamma$  selectively in healthy men.

## 1. Introduction

The intestine has a dual task to develop tolerance to the dietary antigens and commensal microbiota and to develop immunity against potential pathogens [1]. The gut-associated lymphoid tissue consists of organized tissue (Peyer's patches, mesenteric lymph nodes) and diffused tissue (immunoglobulin A-producing plasma cells, mature T cells) and contains the largest mass of immune cells in the human body [1, 2]. The tolerance and defense systems are not accomplished by intestinal immune system alone, but demand the cooperation between epithelial barrier and gut microbiota [1–3]. Intestinal microbiota modulates immune system either through pathogen-associated molecular patterns on the surface of microbes, such as lipopolysaccharides and

peptidoglycans [2], or through microbial products, such as short-chain fatty acids, interacting with both immune cells and enterocytes [4, 5].

Probiotics and prebiotics are potentially important regulators of immune response and have been used in the prevention and treatment of the immune-mediated disorders such as allergies. The criteria of probiotics, (live microorganisms which when administered in adequate amounts confer a health benefit on the host) [6], is fulfilled most of all by several species of lactobacilli and bifidobacteria [7]. Prebiotics are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon, and thus improve host health” [8]. Prebiotics most widely used belong to two chemical groups,

galactooligosaccharides (GOSs) and inulin-type fructans [9]. Probiotics may affect immune system directly [10] or by modifying microbial environment or the processing of antigens in the gut [11]. It is clear that there are differences in immunological effects between probiotic species and strains [12]. Prebiotics may modulate the immune system directly through ligation of carbohydrate or pattern recognition receptors on immune and epithelial cells [5, 9]. More likely, however, prebiotics affect the immune response indirectly by enhancing intestinal fermentation and growth of certain microbes [5, 9].

The aim of the present study was to investigate in healthy men the immunomodulatory potential of the four-strain probiotic combination (*Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Propionibacterium freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99) alone and together with GOSs, which stimulate the growth of bifidobacteria and, to a smaller extent, lactobacilli in the colon [13].

## 2. Materials and Methods

**2.1. Participants.** This immunological study was performed as a part of an intervention study evaluating the effects of probiotics and prebiotics on serum enterolactone concentration [14]. Eighteen healthy Finnish men aged 30–60 years (mean 45 years) volunteered to participate in this study. Gender is known to affect the outcome of the immune response [15], and therefore only men were included. Before entering the study, the participants were interviewed for illness, medication, diet, and smoking. Exclusion criteria included antibiotic treatment for four weeks before the intervention, chronic gastrointestinal diseases, infections of urinary tract with antibiotic treatment, and the use of chemotherapeutics. Two participants used medication for high blood pressure, one for heart disease, one for asthma, and five of the participants smoked. All participants consumed omnivorous diet. The study protocol had been approved by the Ethics Committee of the Foundation for Nutrition Research, Helsinki, Finland, and was carefully explained to the participants, who then gave their written informed consent.

**2.2. Intervention.** This immunological study consisted of a 3-week pretreatment period and a 4-week intervention period. Intervention consisted of two 2-week periods given in a fixed order: (1) juice with probiotic bacteria (65 mL/day), (2) juice with probiotic bacteria (65 mL/day) and GOS (3.8 g/day). During the study, the subjects were not allowed to eat products containing probiotic bacteria and were instructed not to change their ordinary diet. The probiotic juice (Valio Ltd., Helsinki, Finland) contained two *Lactobacillus rhamnosus* strains, GG (ATCC 53103) and LC705 (DSM7061), one bifidobacterium strain, *Bifidobacterium breve* Bb99 (DSM 13692), and one propionibacterium strain, *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM7067), total amount  $2 \times 10^{10}$  viable bacteria daily. After the probiotic juice period, 10.3 g GOS-syrup (Valio Ltd) was added to probiotic juice. It contained 37% of pure GOS resulting in a daily amount of 3.8 g GOS per subject.

**2.3. Data Collection.** Heparinized blood samples for this immunological study were collected at the end of the pretreatment period, probiotics period, and probiotics+GOS period. The sampling was performed in the morning and always on the same day of the week. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Ficoll centrifugation and plasma was collected and stored at  $-20^{\circ}\text{C}$  until analysis. Plasma concentrations of interleukins IL-2, IL-4, IL-5 and IL-10 as well as tumour necrosis factor- $\alpha$  (TNF $\alpha$ ) and interferon- $\gamma$  (IFN $\gamma$ ) were analyzed at baseline as well as at the end of probiotics and probiotics+GOS periods by using a bead-based immunoassay (BD, San Jose, CA, USA). Secretion of IL-4, IL-5, transforming growth factor- $\beta$ 1 (TGF $\beta$ 1) and IFN $\gamma$  by PBMC and their proliferation were assessed at baseline and at the end of the probiotic+GOS period, but not at the end of probiotics period. For the cytokine secretion assay, PBMC were stimulated with either phytohemagglutinin (PHA) 5  $\mu\text{g}/\text{mL}$  or medium alone in U-bottomed wells (Nunc, Roskilde, Denmark) at the density of 200,000 cells in 200  $\mu\text{L}$  of RPMI medium (Gibco, Paisley, UK) containing 5% inactivated AB<sup>+</sup> serum (Finnish Red Cross, Helsinki, Finland) and L-glutamine. The medium was collected after 48 h and the cytokines IL-4, IL-5, TGF $\beta$ 1, and IFN $\gamma$  were assessed by ELISA. Commercial kits were used when analyzing IL-4 (PeliKine Compact, CLB, Amsterdam, the Netherlands) and TGF $\beta$ 1 (Quantikine, R&D Systems, Minneapolis, Minn, USA). IL-5 and IFN $\gamma$  were analyzed as previously described [16, 17]. For the proliferation assay, PBMC were cultured at the density 100 000 cells per 200  $\mu\text{L}$  medium either with or without PHA 5  $\mu\text{g}/\text{mL}$  for 72 h, the cells were harvested 16 h after adding radioactive thymidine (Amersham, Aylesbury, UK), and the incorporation of radioactive thymidine into DNA (cpm) was measured as previously described [18].

**2.4. Statistical Analysis.** The Cochran's Q test was used to compare the percentages of positive (above the detection limits) serum cytokines during pretreatment period, probiotic period and probiotic+GOS periods. In case of positivity of 50% or more, the mean concentrations were compared using the ANOVA of repeated measures. The values below the detection limit were first replaced by "detection limit/2." The distributions of serum cytokines were skewed to the right and were logarithmically transformed before the analysis. Multiple comparisons were not performed as the global tests were nonsignificant for all cytokines. The Wilcoxon-signed ranks test was used to compare probiotic+GOS to the pretreatment period with respect to spontaneous and PHA-stimulated proliferation of PBMC and cytokine production *in vitro*. The data were analyzed using PASW version 18.0 (SPSS Inc. Chicago, Ill, USA).

## 3. Results

All the participants completed the study and none of them used antibiotics during the study.

The proliferation of spontaneous as well as PHA-stimulated PBMC increased during the consumption of probiotics+GOS when compared to the baseline (Figure 1).

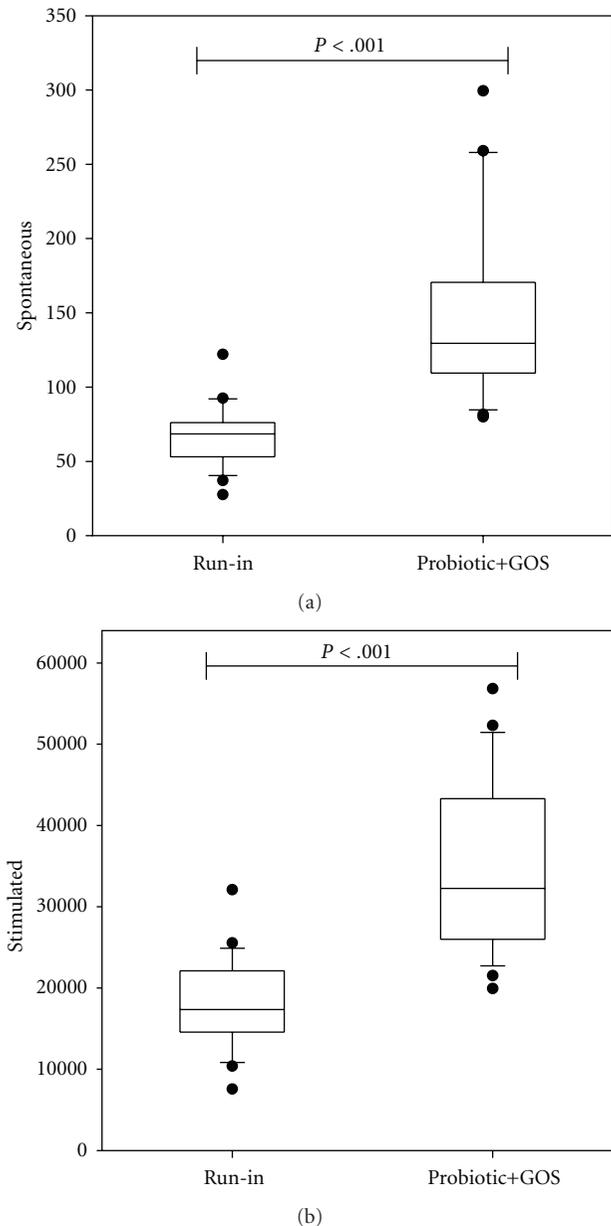


FIGURE 1: Box-blot figures for spontaneous (a) and PHA-stimulated (b) proliferation of PBMC during a pretreatment (run-in) period and during 2 weeks of treatment with the probiotic juice with GOS.  $n = 18$ . Wilcoxon signed ranks test. The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles. The outliers are given as circles.

The percentage increase was 133% for the spontaneous proliferation and 104% for the PHA-stimulated. The secretion of IFN $\gamma$  by PHA-stimulated PBMC increased during the same period (Table 1). The secretions of IL-4, IL-5, and TGF $\beta$ 1 by PBMC were not significantly affected.

Plasma concentrations of IL-2, IL-4, IL-5, IL-10, TNF $\alpha$ , and IFN $\gamma$  were analyzed at baseline and during the probiotics period and the probiotics+GOS period. No significant chan-

ges were found in percentages of positive (above the detection limit) serum cytokines or in mean concentrations of IL-4, IL-5, and IFN $\gamma$  during the study (Table 2). The concentration of IL-2 was below detection limit in more than 50% of the participants during each period and of IL-10 and TNF $\alpha$  during two periods, and therefore the mean values are not presented.

#### 4. Discussion

In the present study, proliferation response of PBMC, both spontaneous and mitogen-stimulated, as well as their mitogen-stimulated IFN $\gamma$  secretion significantly increased during consumption of the probiotic mixture together with GOS in healthy men. The secretion of other cytokines measured was not significantly affected nor were the serum concentrations of any cytokines measured, which indicates a selective response. IFN $\gamma$  is not only antiviral and antimicrobial agent with a broad spectrum, but also an important regulator of overall inflammatory responses [19]. Defective IFN $\gamma$  production in the infancy has been associated with the development of IgE-mediated allergy, and IFN $\gamma$ , and other Th1 lymphocytes, may counteract the Th2 responses of allergy [20]. In fact, the increase of IFN $\gamma$  secretion by stimulated PBMC has been correlated with clinical improvement in children with atopic dermatitis or cow's milk allergy [21]. This kind of effect could explain the reduced eczema and increased resistance to respiratory infections in infants with high risk for allergy by the same probiotic mixture administered together with GOS as used in the present study [22, 23].

The same probiotic mixture as used in the present study (without GOS) did not have a significant effect on IFN $\gamma$  secretion in stimulated PBMC in earlier studies in infants with atopic dermatitis and cow's milk allergy [17]. Similarly, a single-strain consumption of *L. rhamnosus* GG or *Propionibacterium freudenreichii* ssp. *shermanii* did not affect serum IFN $\gamma$  concentration or IFN $\gamma$  production by stimulated PBMC in healthy adults [24]. In children with atopic dermatitis or cow's milk allergy, *L. rhamnosus* GG consumption increased IFN $\gamma$  secretion by stimulated PBMC [17], but it did not affect lymphocyte proliferation in infants [25] nor proliferative responses of stimulated PBMC in supplemented mothers or in their neonates [26]. Therefore, the effects on IFN $\gamma$  production in the present study do not seem to be due to *L. rhamnosus* GG alone. It is possible that GOSs are needed to enhance the colonization of the probiotic bacteria or the immunomodulatory effects of prebiotics and probiotics are additive, as there is data suggesting they enhance the effects of probiotics on the modulation of intestinal environment [14, 27]. GOS supplementation alone was reported recently to reduce the days of cold or flu and stress-induced gastrointestinal dysfunction in healthy young adults [28]. On the other hand, another prebiotic, a mixture of raftilose and raftiline, did not have any effect on IFN $\gamma$  production, or other immune responses, in the elderly [29].

There are numerous studies on the effects of different probiotics and their combinations on the immune system in different ages and diseases, and the results are very different from study to study [12]. The potentially beneficial effects

TABLE 1: Cytokine production *in vitro* by PBMC with or without stimulation by PHA during a pretreatment period and 2 weeks of treatment with probiotic juice with GOS.

Variables	Pretreatment	Probiotics+GOS	P-value <sup>a</sup>
	Median (IQR)	Median (IQR)	
IL-4 spont	0.20 (0.00–1.58)	0.55 (0.00–1.23)	.638
IL-4 PHA <sup>+</sup>	45.3 (31.9–85.7)	38.7 (25.8–68.1)	.255
IL-5 spont	5.0 (0.0–18.8)	14.0 (0.0–31.0)	.156
IL-5 PHA <sup>+</sup>	229 (148–458)	312 (212–621)	.223
IFN $\gamma$ spont	53.0 (0.0–152.0)	43.4 (0.0–116.8)	.508
IFN $\gamma$ PHA <sup>+</sup>	92100 (42300–218000)	267000 (111000–503000)	.022
TGF $\beta$ 1 spont	2460 (918–3750)	2750 (1920–4370)	.122
TGF $\beta$ 1 PHA <sup>+</sup>	3370 (2280–4240)	3720 (2460–6270)	.094

pg/mL.  $n = 18$  for all variables except IFN $\gamma$  spont  $n = 14$ . <sup>a</sup>Wilcoxon signed ranks test.

TABLE 2: Serum cytokines during a pretreatment period, 2 weeks of treatment with probiotic juice, and 2 weeks of treatment with probiotic juice with GOS.

Variables	n (%) above detection limit			P-value <sup>b</sup>	Geometric mean (95% CI) <sup>a</sup>			P-value <sup>c</sup>
	Pretreatment	Probiotic	Probiotic+GOS		Pretreatment	Probiotic	Probiotic+GOS	
IL-4	12 (66.7)	11 (61.1)	15 (83.3)	0.236	2.35 (1.36–4.07)	2.03 (1.22–3.39)	2.56 (1.65–3.99)	0.447
IL-5	12 (66.7)	12 (66.7)	13 (72.2)	0.819	1.21 (0.86–1.71)	1.14 (0.83–1.57)	1.30 (0.95–1.77)	0.415
IFN $\gamma$	9 (50.0)	11 (61.1)	12 (66.7)	0.174	5.96 (2.23–15.94)	5.47 (2.30–13.00)	6.56 (2.84–15.15)	0.441
IL-2	6 (33.3)	6 (33.3)	8 (47.1)	0.449				
IL-10	6 (33.3)	5 (27.8)	9 (50.0)	0.236				
TNF $\alpha$	7 (38.9)	9 (50.0)	8 (44.4)	0.549				

pg/mL.  $n = 18$ . <sup>a</sup>Geometric means are given if at least half of observations are above the detection limit. Values below the detection limit were replaced by “detection limit/2”. <sup>b</sup>Cochran’s Q test. <sup>c</sup>ANOVA for repeated measures using logarithmically (ln) transformed values.

of several probiotics on immune system, such as increased IFN $\gamma$  secretion by stimulated PBMC, have been reported in allergic children [17, 21, 30, 31], whereas their effects in healthy adults are not as often shown: *L. casei* strain Shirota, *L. casei* DN-114001 nor *B. animalis* ssp. *lactis* Bb12 together with *L. paracasei* ssp. *paracasei* CRL-431, have not significantly affected production of IFN $\gamma$  [32–35]. In a small study in rhinopathic patients, the combination of *L. acidophilus* and a *bifidobacterium* strain did not affect PBMC proliferation [36]. However, *L. acidophilus* LAFTI L10 consumption slightly increased IFN $\gamma$  secretion from T cells in fatigued athletes and IFN- $\gamma$  concentration in saliva in healthy athletes [37]. Our results are supported by a previous study where a consumption of prebiotic inulin enriched with oligofructose in combination with the probiotics *L. rhamnosus* GG and *B. lactis* Bb12 increased the capacity of stimulated PBMC to selectively produce IFN $\gamma$  in resected colon cancer patients [38].

The present study design with sequential interventions has disadvantages. The randomized controlled trial (RCT) such as parallel groups with three groups or four groups (i.e., factorial design) or crossover study with randomized order of treatments were not possible for practical reasons. Thus, in the present study, the time-dependent covariates and carry-over effects are possible sources of bias and may reduce the differences between treatments.

## 5. Conclusions

The consumption of this specific four-strain probiotic combination together with GOS stimulated the *in vitro* PBMC proliferation and IFN $\gamma$  production. These effects may be beneficial in states where IFN $\gamma$  production is insufficient, such as in allergies.

## Abbreviations

GOS: Galactooligosaccharides  
 IFN $\gamma$ : Interferon- $\gamma$   
 IL-4: Interleukin-4  
 IL-5: Interleukin-5  
 PBMC: Peripheral blood mononuclear cells  
 PHA: Phytohemagglutinin  
 TGF $\beta$ 1: Transforming growth factor- $\beta$ 1  
 IL-2: Interleukin-2  
 IL-10: Interleukin-10  
 TNF $\alpha$ : Tumour necrosis factor- $\alpha$ .

## Disclosure

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Finland. R. Korpela is a former employee of Valio Research Centre.

## Conflict of Interests

R. Holma, T. Poussa, O. Vaarala, and H. Adlercreutz have no conflicts of interests.

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