

## Consumption of prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* has minor effects on selected immune parameters in polypectomised and colon cancer patients

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Probiotics (PRO) modulate immunity in humans, while the effect of prebiotics (PRE) and synbiotics (SYN) on the human immune system are not well studied yet. The objective of this study was to investigate whether daily intake of a SYN modulates immune functions. In a randomised double-blind, placebo-controlled trial, thirty-four colon cancer patients who had undergone ‘curative resection’ and forty polypectomised patients participated. Subjects of the SYN group daily received encapsulated bacteria ( $1 \times 10^{10}$  colony-forming units of *Lactobacillus rhamnosus* GG (LGG) and  $1 \times 10^{10}$  colony-forming units of *Bifidobacterium lactis* Bb12 (Bb12)) and 10 g of inulin enriched with oligofructose. Controls received encapsulated maltodextrin and 10 g of maltodextrin. Prior to intervention (T1), and 6 (T2) and 12 weeks after the start of the intervention (T3), phagocytic and respiratory burst activity of neutrophils and monocytes, lytic activity of natural killer cells and production of interleukin (IL)-2, IL-10 and IL-12, as well as tumour necrosis factor- $\alpha$  and interferon- $\gamma$  (IFN- $\gamma$ ) by activated peripheral blood mononuclear cells (PBMC) were measured. In faeces, the concentrations of transforming growth factor- $\beta$ 1 and prostaglandin E<sub>2</sub> were measured. IL-2 secretion by activated PBMC from the polyp group increased significantly between T1 or T2 and T3 ( $P < 0.05$ ). In the cancer group, SYN treatment resulted in an increased capacity of PBMC to produce IFN- $\gamma$  at T3 ( $P < 0.05$ ). Other immunity-related parameters were not affected by SYN treatment, neither in the cancer nor in the polyp group. In conclusion, supplementation with this SYN has minor stimulatory effects on the systemic immune system of the two study groups. Further studies in humans should aim to focus on the gut-associated immune system.

**Randomised double blind placebo-controlled trial: Immune system: Human: Prebiotic: Probiotic: Synbiotic**

Numerous studies in animals and to a lesser extent in humans have investigated the immunomodulatory effects of probiotics (PRO) (Erickson & Hubbard, 2000; Meydani & Ha, 2000; Gill & Cross, 2002). In humans, the oral intake of PRO increased *ex vivo* cytokine (interferon- $\alpha$  (IFN- $\alpha$ )) secretion of peripheral blood mononuclear cells (PBMC) (Kishi *et al.* 1996; Arunachalam *et al.* 2000). Consumption of probiotics changed the relative proportions of leucocyte subsets and enhanced phagocytic activity as well as natural killer (NK) cell cytotoxicity (Gill *et al.* 2001; Olivares *et al.* 2006). The results of these studies, however, vary greatly due to the diversity of the investigated microorganisms and usage of different experimental models (Bourlioux *et al.* 2003; Christensen *et al.* 2006). The effects of prebiotics (PRE) on the human immune system are not well studied. A comprehensive

overview of the experimental findings is given in two recent reviews (Schley & Field, 2002; Watzl *et al.* 2005). Currently, only a few human intervention studies have looked at the immunomodulatory effects of PRE alone. In one study with elderly people, supplementation with fructooligosaccharides increased the percentage of CD4<sup>+</sup> and CD8<sup>+</sup>T cells, whereas the phagocytic activity of granulocytes and monocytes was decreased as well as expression of interleukin (IL)-6 mRNA in monocytes. However, this study did not include a placebo control (Guigoz *et al.* 2002). In patients with Crohn’s disease, PRE stimulated the percentage of IL-10-positive dendritic cells in the intestinal mucosa (Lindsay *et al.* 2006).

A synbiotic (SYN) is defined as a combination of a PRO and a PRE. Only limited data are available about the immunomodulatory potential of SYN. The SYN combination used in

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**Abbreviations:** Bb12, *Bifidobacterium lactis* Bb12; IFN, interferon; IL, interleukin; LGG, *Lactobacillus rhamnosus* GG; NK, natural killer; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PRE, prebiotics; PRO, probiotics; SYN, synbiotics; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; TNF- $\alpha$ , tumour necrosis factor- $\alpha$ .

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the present study has been investigated in a short-term and a long-term animal study. Feeding rats for 4 weeks with the SYN increased secretory immunoglobulin A production in the ileum (Roller *et al.* 2004b). In the long-term study, azoxymethane-treated rats were fed for 33 weeks with the SYN and the animals were evaluated for the induction of colon cancer. SYN supplementation influenced primarily the immune cells of Peyer's patches, increasing their NK cell-like activity and their capacity to produce IL-10 (Roller *et al.* 2004a). Significantly fewer tumours occurred in the SYN- and PRE-fed rats compared with controls (Femia *et al.* 2002).

The present study examined the effects of the PRE inulin enriched with oligofructose in combination with the PRO *Lactobacillus rhamnosus* GG (LGG) and *Bifidobacterium lactis* Bb12 (Bb12) on the immune system of polypectomised human subjects and of resected colon cancer patients. We used two PRO strains because others have suggested that a combination of PRO may have a greater effect on the intestine than the individual strains (Campieri & Gionchetti, 1999; Ouwehand *et al.* 2000). Polypectomised and previously resected colon cancer patients were recruited because the overall aim of the EU-funded project was to identify new cancer risk biomarkers and to study whether SYN may reduce colon cancer risk. Immune parameters of non-specific host resistance including immune functions which are relevant for tumour cell recognition and elimination, such as the lytic activity of NK cells, were primarily analysed. Because intestinal biopsy material providing immune cells were not available, immune cells from peripheral blood were investigated.

## Subjects and methods

### Subjects

Subjects were recruited from the patient lists of consultant gastrointestinal surgeons in The Mercy University Hospital who fulfilled the inclusion and exclusion criteria of the study. Inclusion criteria were (1) biopsy- and histologically confirmed spontaneous but not familial adenomatous polyps within the last 5 years or (2) undergone resection for histologically confirmed colon cancer within the last 5 years. Exclusion criteria were age over 75 years, pregnancy, known lactose intolerance, clinically significant immunodeficiency, usage of antibiotics and additional gastrointestinal disorders (e.g. Crohn's disease or ulcerative colitis). Details of the inclusion and exclusion criteria are reported elsewhere (van Loo *et al.* 2005). A total of thirty-seven colon cancer patients were recruited, of which thirty-four patients completed the trial; thirteen were females and twenty-one were males, with an age of 60.1 (SD 5.8) and 62.1 (SD 5.3) (mean and SD), respectively. In this group, fifteen subjects received placebo and nineteen subjects received SYN treatment. A total of forty-three polypectomised patients (polyp patients) were recruited, of which forty patients completed the trial; eighteen were females and twenty-two were males, with an age of 56.0 (SD 9.8) and 58.0 (SD 9.7) (mean and SD), respectively. In this group, twenty-one subjects received placebo and nineteen subjects received SYN treatment. Prior to the recruitment, the study was evaluated and approved by the Cork University Hospitals Ethics Committee.

### Study design

This study was a randomised double-blind and placebo-controlled study. Subjects were randomly assigned to the SYN or placebo group. Patients in the SYN group received daily encapsulated bacteria ( $1 \times 10^{10}$  colony-forming units of LGG and  $1 \times 10^{10}$  colony-forming units of Bb12) and a 10 g sachet of inulin enriched with oligofructose. An acid-resistant coating was used to prepare the capsules containing the PRO. Patients in the placebo group received daily encapsulated maltodextrin and a 10 g sachet of maltodextrin. The intervention period lasted 12 weeks. Subjects kept a 6-week diary for each phase of the intervention. Prior to the intervention (T1) and at week 6 (T2), the subjects received a numbered box containing sufficient product for 6 weeks. At T2 and after 12 weeks (T3), subjects were interviewed by the study nurse, and reactions to the product, medications taken and any adverse events that had occurred in each 6-week period were recorded. The amount of product returned was recorded to confirm compliance. Blood and faecal samples were obtained prior to intervention, and at 6 and 12 weeks after the intervention. During the study period, subjects were asked to adhere to their usual diets. Subjects were instructed to take the SYN/placebo supplement with their main meals to minimise the inhibitory effects of gastric acid on the PRO.

For logistical reasons, the isolation, stimulation and cryopreservation of PBMC took place in Cork. Phagocytic activity and oxidative burst of blood cells was measured in Cork. Preparation of faecal water was also carried out in Cork. Supernatants of stimulated cells, cryopreserved cells and faecal water samples were stored frozen in Cork until the end of the study, and then sent all at once by overnight courier on dry ice from Cork, Ireland, to Karlsruhe, Germany.

### Materials

LGG was provided by Valio (Helsinki, Finland) and Bb12 was purchased from Ch. Hansen (Horsolm, Denmark). Raftilose Synergy1<sup>®</sup> was provided by Orafiti (Tienen, Belgium). Raftilose Synergy1<sup>®</sup> is a 1:1 mixture of long-chain and short-chain fractions of inulin, a  $\beta(2-1)$ -fructan extracted from chicory (*Cichorium intybus*) roots.

### Isolation of PBMC

Blood was drawn into tubes containing Li-heparin (Sarstedt, Nümbrecht, Germany). PBMC were isolated by density gradient centrifugation using Histopaque 1077 (Sigma, Deisenhofen, Germany) and resuspended in complete RPMI-1640 culture medium (Life Sciences, Eggenstein-Leopoldshafen, Germany), containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mmol/l), penicillin (100 000 U/l) and streptomycin (100 mg/l).

### Cryopreservation of lymphocytes

Isolated PBMC were centrifuged at 400g for 8 min at 20°C. Cells were resuspended in freezing medium (70% RPMI-1640, 20% fetal bovine serum (FBS), 10% dimethylsulphoxide) at a concentration of  $2 \times 10^{10}$  cells/l. A 300  $\mu$ l aliquot of the cell suspensions was placed into cooled cryogenic freezing

vials and put in a cryopreservation vessel overnight at  $-80^{\circ}\text{C}$ . After 24 h, freezing vials were transferred to liquid nitrogen in which they were stored until sending them on dry ice to Karlsruhe (Germany). In Karlsruhe, cells were stored overnight at  $-80^{\circ}\text{C}$  and then transferred to liquid nitrogen until the time of analysis.

#### *Lytic activity of NK cells*

Cryopreserved PBMC were defrosted in a  $37^{\circ}\text{C}$  water bath, suspended in 10 ml of complete RPMI-1640 culture medium, centrifuged (400 g, 10 min,  $20^{\circ}\text{C}$ ) and resuspended in complete medium (Fujiwara *et al.* 1986). PBMC were incubated for 18 h at  $37^{\circ}\text{C}$ . After washing the cells with complete medium, they were diluted to  $2.5 \times 10^9$  cells/l and used for determination of lytic activity of NK cells against K-562 cells (effector-target ratio 12.5:1) as described (Chang *et al.* 1993). A flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany) was used to determine the percentage of killed target cells.

#### *Phagocytic activity and intensity*

Assessment of phagocytic activity (percentage of phagocytic-active cells) and phagocytic intensity (number of phagocytised *Escherichia coli* per phagocytic cell expressed as mean fluorescence) was based on a described flow cytometric method (O'Gorman, 2002). Briefly, to measure phagocytic activity, 10  $\mu\text{l}$  of opsonised BODYPY FL-labelled *E. coli* (Molecular Probes, Leiden, The Netherlands) and 100  $\mu\text{l}$  of fresh whole blood were mixed and incubated at  $37^{\circ}\text{C}$  for 10 min. The reaction was stopped by adding 100  $\mu\text{l}$  of ice-cold quenching solution (10% trypan blue in phosphate-buffered saline (PBS)). After washing, whole blood cells were fixed with 2 ml of 1  $\times$  lysing solution (BD, Heidelberg, Germany). DNA was stained with 300  $\mu\text{l}$  of propidium iodide solution (33.3 mg/l dissolved in PBS). A flow cytometer (Epics Elite, Beckman Coulter, Fullerton, CA) was used to measure the level of phagocytic activity and intensity in neutrophils and monocytes.

#### *Respiratory burst*

Fresh whole blood (100  $\mu\text{l}$ ) was cooled on ice to  $0^{\circ}\text{C}$ . Tubes were incubated with 20  $\mu\text{l}$  of PBS (control) or 20  $\mu\text{l}$  of phorbol myristate 13-acetate (8.1  $\mu\text{mol/l}$ ; Sigma-Aldrich) as a high stimulus for 10 min at  $37^{\circ}\text{C}$ . A 1.7  $\mu\text{l}$  aliquot of dihydrorhodamine 123 (10 g/l in dimethylsulphoxide; Molecular Probes, Leiden, The Netherlands) was added and samples were incubated for 15 min at  $37^{\circ}\text{C}$ . Erythrocytes were lysed with 2 ml of 1  $\times$  lysing solution. After washing, cells were resuspended in 300  $\mu\text{l}$  of propidium iodide solution (33.3 mg/l dissolved in PBS) and incubated for 10 min on ice. A flow cytometer (Epics Elite, Beckman Coulter) was used to measure the percentage of neutrophils that produced reactive oxygen species and also the intensity of the production.

#### *Quantification of cytokine secretion*

PBMC isolated from fresh whole blood were suspended at a concentration of  $1 \times 10^9$  cells/l in complete medium and were stimulated with mitogens for 24 h at  $37^{\circ}\text{C}$ . PBMC

were stimulated by 5 mg/l concanavalin A (Sigma-Aldrich) (IL-2/IFN- $\gamma$ ), by 1  $\mu\text{g/l}$  lipopolysaccharide (*E. coli* 0111:B4, BD) (tumour necrosis factor- $\alpha$  (TNF- $\alpha$ /IL-12) or by 1 mg/l phytohaemagglutinin (lectin of *Phaseolus vulgaris*, Sigma Aldrich) (IL-10). Cell-free supernatants were collected and stored at  $-80^{\circ}\text{C}$  until analysis. IL-2 was measured by sandwich-enzyme-linked immunosorbent assay (ELISA) using an anti-human IL-2 monoclonal antibody (3 mg/l in PBS, R&D Systems, Wiesbaden, Germany) as capture antibody and a biotin-labelled anti-human IL-2 monoclonal antibody (50  $\mu\text{g/l}$  1 PBS-Tween and 2% v/v bovine serum albumin, R&D Systems) as detection antibody. Recombinant human IL-2 (R&D Systems) was used as standard. Levels of IL-10, IL-12, TNF- $\alpha$  and IFN- $\gamma$  in the supernatants were measured with CytoSets™ commercial ELISA kits (Biosource, Solingen, Germany).

#### *Faecal water preparation*

Total faeces of one passage from the patients were collected in the early morning and stored cool until preparation. Aliquots of the total faeces were diluted with ice-cold Dulbecco's modified Eagle's medium (DMEM; 1 g of faeces +1 ml of DMEM) and homogenised in a stomacher for 2 min. The homogenates were centrifuged at 60 000 g for 2 h at  $4^{\circ}\text{C}$ . The resultant supernatants were filtered through a 0.2  $\mu\text{m}$  filter with a glass pre-filter (Nalgene, Fisher Scientific, Schwerte, Germany). Aliquots of 1 ml were stored at  $-80^{\circ}\text{C}$  prior to analysis.

#### *Quantification of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in faecal water*

Sample purification was done with a C18 minicolumn (Amprep C18 RPN1910; 500 mg/2.8 ml; Amersham, Freiburg, Germany). Faecal water samples were centrifuged for 5 min at 2500 g ( $20^{\circ}\text{C}$ ). A 100  $\mu\text{l}$  aliquot of the supernatants was diluted 1:10 with distilled H<sub>2</sub>O and passed through a 0.2  $\mu\text{m}$  filter. A 500  $\mu\text{l}$  aliquot of the filtered sample was mixed with ice-cold 500  $\mu\text{l}$  water-ethanol solution (1:4) and 10  $\mu\text{l}$  of glacial acetic acid, and incubated for 5 min at  $20^{\circ}\text{C}$  followed by centrifugation (2500 g, 5 min,  $20^{\circ}\text{C}$ ). C18 minicolumns were primed with 5.6 ml of 10% ethanol. Supernatants of the samples were applied to the columns which were then washed with 2.8 ml of distilled H<sub>2</sub>O and with 2.8 ml of hexane. PGE<sub>2</sub> was eluted with 3 ml of ethyl acetate. Ethyl acetate was evaporated in a vacuum centrifuge. Pellets were stored at  $-80^{\circ}\text{C}$  prior to analysis. The levels of PGE<sub>2</sub> were measured with a commercial EIA kit (Amersham). For measurement, pellets were resuspended with 250  $\mu\text{l}$  of assay buffer.

#### *Quantification of transforming growth factor- $\beta$ (TGF- $\beta$ 1) in faecal water*

For activation of latent TGF- $\beta$ 1, faecal water samples were centrifuged (2500 g, 5 min,  $20^{\circ}\text{C}$ ). An 80  $\mu\text{l}$  aliquot of the supernatants was mixed with 80  $\mu\text{l}$  of acetic acid (2.5 mol/l)/urea (10 mol/l) and incubated ( $20^{\circ}\text{C}$ , 10 min). Samples were neutralized with 80  $\mu\text{l}$  of NaOH (2.7 mol/l)/HEPES (1 mol/l). Levels of TGF- $\beta$ 1 were immediately measured with a commercial ELISA kit (R&D Systems).

### Statistical analyses

Normal distribution of the data was analysed by using the Kolmogorov–Smirnov normality test. We used repeated measures analysis of variance (ANOVA) to analyse changes of the various immune parameters. Treatment (placebo or SYN) was included as main effect, and change in immune parameters over time as dependent variables. Study group (cancer *v.* polyp) as a main effect was not included in the ANOVA due to significant differences in various immunological markers at baseline. When the interaction was significant, *post hoc* analysis of the effect of treatment group was performed by one-factor ANOVA with Dunnett's test. All statistical calculations were performed with the PROC Mixed procedure in SAS Software (version 6.12) (SAS Institute, Cary, NC, USA). Values of  $P < 0.05$  were considered significant.

### Results

Three cancer subjects withdrew; each of them was in the placebo group. One subject withdrew due to a chest infection, another subject felt unwell on the product and the third subject was diagnosed with nephrotic syndrome unassociated with the product. Two polyp subjects in the SYN group withdrew; one subject moved from Cork and the second subject withdrew due to bloating associated with the product. One polyp subject in the placebo group withdrew following diagnosis with temporal arthritis which was unrelated to the product.

In a pilot study, levels of LGG and Bb12 in faeces were measured before and after the SYN treatment. In this pilot study, up to 10% of the lactic acid bacteria provided with the SYN survived passage through the gastrointestinal tract (van Loo *et al.* 2005).

The percentages of phagocytic active neutrophils and monocytes and their phagocytic intensity were not modulated by the dietary intervention in either the cancer or polyp group (Table 1). The SYN treatment did not affect the percentage of neutrophils that produced reactive oxygen species and the intensity of the production in both study groups (Table 1). Lytic activity of NK cells was not significantly changed by the intake of the SYN in both groups (Table 1).

The capacity to produce IL-2 by activated PBMC from the polyp group differed significantly between the placebo and SYN group at T3 (Table 2). While in the placebo group the IL-2 secretion increased at T3, this effect did not occur in the SYN-supplemented polyp group. In contrast, in the cancer group, subjects' IL-2 secretion was not affected by SYN treatment. There was no significant difference in production of the cytokines IL-10, IL-12 and TNF- $\alpha$  due to the intervention in either the cancer or polyp group (Table 2). However, in the cancer group, the treatment with SYN significantly increased the IFN- $\gamma$ -producing capacity of PBMC at T3 compared with T2 (Table 2). This effect was not observed with subjects from the polyp group.

The intake of the SYN did not affect the concentration of PGE<sub>2</sub> and TGF- $\beta$ 1 in faecal water in either the cancer or the polyp group (Table 3).

### Discussion

Our study was done in the context of a project funded by the Commission of the European Communities with the aim to

identify new cancer risk biomarkers and to determine whether SYN can reduce the risk for colon cancer. The role of the present study was to investigate the impact of SYN on the immune system, which potentially mediates a protective effect of the SYN supplement on colon carcinogenesis. The immune system plays an important role in the control of tumour promotion and progression. The generation of an effective anti-tumour immune response depends on close interaction of several elements of the immune system. These include antigen-presenting cells, and different subsets of T cells, B cells and NK cells (Gabilovich & Pisarev, 2003). Considerable evidence is available from animal and *in vitro* studies that PRO (Rafter, 2003), and to a lesser extent PRE (Delzenne *et al.* 2003), have the potential to reduce colon cancer risk. Data from animal studies would suggest that using a combination of PRO and PRE may be the most effective strategy to maximise any anti-carcinogenic effects in the intestinal tract (Burns & Rowland, 2000; Femia *et al.* 2002). Data from human studies (epidemiology and intervention), however, are rare. Until now, only one human study investigated the effect of a SYN in patients with active ulcerative colitis. The outcome of this study suggests that this SYN (containing a similar PRE to that in the present study) reduced intestinal inflammation (Furie *et al.* 2005). Therefore, we investigated immunomodulatory effects of a daily SYN intake in resected colon cancer patients who had no intervention other than surgery, and subjects considered at increased risk for colon cancer (polypectomised patients).

In the present study, the intake of the SYN did not affect phagocytic activity, respiratory burst and the lytic activity of NK cells in either study group. Two other studies have looked at the effect of the combined application of PRO with the PRE galactooligosaccharide (Chiang *et al.* 2000; Sheih *et al.* 2001). In contrast to our findings, galactooligosaccharide in combination with *B. lactis* enhanced NK cell activity compared with the PRO alone, while galactooligosaccharide in combination with *L. rhamnosus* HN001 was not significantly different from the PRO alone. Intake of the PRO alone also increased phagocytic activity in both studies. Neither study included a PRE group alone or was controlled for time effects, so conclusions regarding the immunomodulatory potential of the synbiotic approach cannot be drawn. Consumption of fermented milk containing *L. casei* Shirota for 3 weeks resulted likewise in an enhancement of NK cell activity in subjects with low NK cell activity at baseline (Nagao *et al.* 2000). In another human study with subjects with normal NK cell activity at baseline, intake of *L. casei* Shirota-fermented milk for 4 weeks did not influence NK cell activity, phagocytic activity or respiratory burst (Spanhaak *et al.* 1998) which is in line with our results. Presumably, differences in the study design, study subjects and immune status at baseline may in part explain the different findings.

A number of lymphocyte and monocyte-specific cytokines in supernatants of *ex vivo*-activated PBMC were measured. In the placebo polyp group, a rise of IL-2 secretion by activated PBMC occurred at T3, whereas in the SYN polyp group IL-2 secretion did not differ between time points. Whether the SYN supplement prevented the increase in IL-2 secretion at T3 and which factors may have caused the increase in the placebo group is currently not known. Subjects

**Table 1.** Immune parameters measured prior to supplementation (T1), and at 6 weeks (T2) and 12 weeks (T3) after supplementation with placebo or synbiotic in two study groups (cancer and polyp group subjects)

(Mean values and standard error of the mean)

Time...	Placebo (n 15)						Synbiotic (n 19)						P values (time × treatment)
	T1		T2		T3		T1		T2		T3		
Cancer patients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Phagocytic activity (%)													
Neutrophils	49	5	38	4	43	5	48	4	35	4	48	4	0.685
Monocytes	22	3	16	2	21	3	21	3	15	2	19	2	0.996
Phagocytic intensity (mean fluorescence per active phagocyte)													
Neutrophils	23	3	18	2	26	3	23	1	17	1	25	2	0.666
Monocytes	28	7	17	3	21	2	22	2	20	5	18	2	0.360
Respiratory burst activity (%)													
Neutrophils	87	2	91	1	90	1	87	2	90	1	91	1	0.378
Respiratory burst intensity (mean fluorescence per active granulocyte)													
Neutrophils	16	1	20	3	21	3	17	1	20	3	25	2	0.441
Lytic activity of NK cells (% lysed K562 target cells; effector–target ratio 12.5:1)	13	2	18	2	15	3	14	2	14	1	15	2	0.328
Time...	Placebo (n 21)						Synbiotic (n 19)						P values (time × treatment)
	T1		T2		T3		T1		T2		T3		
Polyp patients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
Phagocytic activity (%)													
Neutrophils	35	4	38	4	46	4	41	5	40	4	46	5	0.826
Monocytes	17	2	19	2	18	2	19	2	19	3	17	2	0.795
Phagocytic intensity (mean fluorescence per active phagocyte)													
Neutrophils	20	1	18	1	24	3	22	1	20	2	25	3	0.928
Monocytes	19	1	17	1	24	3	24	4	19	2	25	4	0.875
Respiratory burst activity (%)													
Neutrophils	87	1	90	1	90	1	90	1	91	1	87	3	0.355
Respiratory burst intensity (mean fluorescence per active granulocyte)													
Neutrophils	20	3	22	3	29	4	15	1	20	2	20	3	0.508
Lytic activity of NK cells (% lysed K562 target cells; effector–target ratio 12.5:1)	19	2	18	1	22	2	21	3	20	1	18	1	0.125

NK, natural killer.

**Table 2.** Cytokine secretion of activated PBMC isolated from cancer or polyp group subjects prior to supplementation (T1), and at 6 weeks (T2) and 12 weeks (T3) after supplementation with placebo or synbiotic

(Mean values and standard error of the mean)

Time...	Placebo (n 15)						Synbiotic (n 19)						P values (time × treatment)
	T1		T2		T3		T1		T2		T3		
Cancer patients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
IL-2 (ng/l)	260	67	149	34	132	21	235	64	115	18	221	58	0.241
IL-10 (ng/l)	138	30	128	27	135	39	153	25	126	24	123	19	0.889
IL-12 (ng/l)	816	205	618	143	745	124	779	161	665	136	917	167	0.827
TNF-α (μg/l)	8.4	1.9	6.1	0.3	8.6	0.4	9.8	0.3	5.7	1.2	9.0	1.8	0.902
IFN-γ (ng/l)	826	225	741	183	712	151	642	176	504	124	1071	188*	0.053
Time...	Placebo (n 21)						Synbiotic (n 19)						P values (time × treatment)
	T1		T2		T3		T1		T2		T3		
Polyp patients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
IL-2 (ng/l)	168	38	153	38	353	62†	153	27	190	39	171	37	0.013
IL-10 (ng/l)	134	31	121	19	143	22	82	20	141	32	138	23	0.332
IL-12 (ng/l)	414	98	559	132	829	112	341	91	719	155	599	137	0.242
TNF-α (μg/l)	6.9	0.3	9.0	0.3	8.4	2.0	6.8	0.3	8.7	0.3	5.0	1.4	0.740
IFN-γ (ng/l)	850	189	1014	220	1317	187	928	227	1013	227	826	186	0.241

IL, interleukin; TNF, tumour necrosis factor; IFN, interferon.

\* Significantly different between T2 and T3 in synbiotic-treated cancer group subjects ( $P < 0.05$ ).† Significantly different from T1 and T2 in the placebo group of polyp patients ( $P < 0.05$ ).

**Table 3.** Concentration of TGF- $\beta$ 1 ( $\mu$ g/l) and PGE<sub>2</sub> ( $\mu$ g/l) in faecal water of cancer or polyp group subjects prior to supplementation (T1), and at 6 weeks (T2) and 12 weeks (T3) after supplementation with placebo or synbiotic (Mean values and standard error of the mean)

Time...	Placebo (n 15)						Synbiotic (n 19)						P values (time $\times$ treatment)
	T1		T2		T3		T1		T2		T3		
Cancer patients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
TGF- $\beta$ 1	2.5	0.6	2.7	0.7	2.4	0.5	1.0	0.2	1.5	0.5	0.8	0.1	0.436
PGE <sub>2</sub>	32	9	33	9	28	8	24	6	24	6	25	5	0.511

  

Time...	Placebo (n 21)						Synbiotic (n 19)						P values (time $\times$ treatment)
	T1		T2		T3		T1		T2		T3		
Polyp patients	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
TGF- $\beta$ 1	1.8	0.4	1.5	0.3	1.7	0.3	1.4	0.3	2.2	0.5	1.9	0.5	0.133
PGE <sub>2</sub>	13	3	11	4	10	2	10	3	8	2	10	3	0.481

TGF, transforming growth factor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>.

were enrolled for this study over a time period of approximately 1 year. Therefore, seasonal effects can be excluded as a cause for the increase in IL-2.

While the capacity of activated PBMC to produce IFN- $\gamma$  in the polyp group was not significantly affected by the SYN, in the cancer group supplementation with the SYN increased secretion of IFN- $\gamma$  at T3. An increase in IFN- $\gamma$  production in SYN-supplemented cancer patients could be interpreted as a positive effect due to the role of this cytokine in the immunosurveillance of cancer (Smyth *et al.* 2001). A recent human intervention study measured the effect of a SYN (*Bifidobacterium longum*, Synergy 1) on cytokine mRNA expression in rectal biopsies from patients with ulcerative colitis. TNF- $\alpha$  and IL-1 $\alpha$  mRNA expression were significantly reduced after 1 month with SYN, suggesting an anti-inflammatory effect of this SYN (Furrie *et al.* 2005). No other human SYN study measured the effect on cytokine secretion, but several studies with PRO examined IFN- $\gamma$  production of PBMC. In accordance with our results, uptake of yoghurt containing live cultures during a 4-month period increased IFN- $\gamma$  secretion of PBMC (Halpern *et al.* 1991). No information about the bacterial strains used and their dosage is available. The intake of fermented milk with *L. casei* Shirota for 3 or 4 weeks had no influence on IFN- $\gamma$  secretion of activated PBMC and IFN- $\gamma$  concentration in serum (Spanhaak *et al.* 1998; Nagao *et al.* 2000). After ingestion of a PRE mixture (inulin with oligofructose) in combination with a nutritional supplement for 8 weeks, the IFN- $\gamma$ -secretion of activated PBMC was not influenced (Bunout *et al.* 2002) which is in line with our results obtained after 6 weeks. In our study, IFN- $\gamma$  secretion was only affected after an intake of 12 weeks, suggesting that only a long-term intake results in immunomodulation. Again a seasonal effect for the enhanced IFN- $\gamma$  secretion can be excluded. Why the increase in IFN- $\gamma$  secretion was not also seen in polyp group subjects is currently not known. However, immunological data mostly differed between the two groups, indicating that the immunological status among these groups was already different at baseline. Taken together, supplementation with the SYN did not have a strong effect on cytokine secretion by activated PBMC. Moreover, secretion of IL-10, IL-12 and TNF- $\alpha$  was not influenced by the SYN in either group.

Due to limitations in the availability of biopsies within the project, we were not able to isolate immune cells, such as intraepithelial lymphocytes from the intestinal epithelium or Peyer's patch cells, for measuring immune functions in the gut. As a surrogate, in faecal water we quantified concentrations of PGE<sub>2</sub> and TGF- $\beta$ 1, which are produced by intestinal epithelial and immune cells, modulating immune cell functions within the gut, and which reflect intestinal production of these molecules. Levels of PGE<sub>2</sub> have been found to be elevated in human colon cancer samples (Rigas *et al.* 1993; Pugh & Thomas, 1994). PGE<sub>2</sub> is known to promote tumour cell survival and has diverse effects on immune cells (Harris *et al.* 2002), in particular NK cell activity is suppressed by PGE<sub>2</sub> (Rhind *et al.* 1999; Yakar *et al.* 2003). TGF- $\beta$  possesses immunomodulatory activity, acting predominantly in an immunosuppressive way which inhibits cell proliferation of many cell types (Derynck & Choy, 1998). In later stages of cancer development, TGF- $\beta$ 1 contributes to cell growth, invasion and metastasis (Pasche, 2001). Supplementation with the SYN did not affect concentrations of PGE<sub>2</sub> or TGF- $\beta$ 1 in faecal water in either study group.

The mechanisms by which PRO and PRE modulate the immune system are not well understood (Cross, 2002; Perdigon & Raya, 2002; Watzl *et al.* 2005). It is known that the gut microflora interacts with mucosal cells including epithelial cells and underlying immune cells (Marteau *et al.* 2002). *In vitro* assays indicate that cell wall components of lactic acid bacteria activate immune cells such as lymphocytes and macrophages (Meydani & Ha, 2000). Moreover, bacterial DNA activates cells of both the innate and acquired immune systems (Hacker *et al.* 2002). PRE selectively stimulate the growth of beneficial bacteria in the colon (Gibson & Roberfroid, 1995). As a result of this, PRE might modulate the immune system directly by affecting the composition of the intestinal flora. In addition, the fermentation of PRE produces short chain fatty acids in the colon (Flamm *et al.* 2001) which may affect the gut-associated lymphoid tissue (Kvale & Brandtzaeg, 1995; Chapman, 2001; Sanderson, 2004).

In conclusion, no negative effects were observed in cancer and polyp patients with daily consumption of this SYN for 12 weeks, but more definitive testing is required to indicate

conclusively that this product is safe. The SYN supplement had only minor effects on the immune system of the subjects in both groups. It is possible that SYN supplementation in humans preferentially affects the gut-associated lymphoid tissue rather than the systemic immune system. Therefore, future studies with humans and SYN should aim to focus on the effects at the level of the gut-associated lymphoid tissue. Further, we have only tested two strains of lactic acid bacteria; however, other strains could result in a more significant modulation of the immune system.

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### References

- Arunachalam K, Gill HS & Chandra RK (2000) Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). *Eur J Clin Nutr* **54**, 263–267.
- Bourlioux P, Koletzko B, Guarner F & Braesco V (2003) The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium 'The Intelligent Intestine', held in Paris, June 14, 2002. *Am J Clin Nutr* **78**, 675–683.
- Bunout D, Hirsch S, Pia de la Maza M, Munoz C, Haschke F, Steenhout P, Klassen P, Barrera G, Gattas V & Petermann M (2002) Effects of prebiotics on the immune response to vaccination in the elderly. *J Parenter Enteral Nutr* **26**, 372–376.
- Burns AJ & Rowland IR (2000) Anti-carcinogenicity of probiotics and prebiotics. *Curr Issues Intest Microbiol* **1**, 13–24.
- Campieri M & Gionchetti P (1999) Probiotics in inflammatory bowel disease: new insight to pathogenesis or a possible therapeutic alternative? *Gastroenterology* **116**, 1246–1249.
- Chang L, Gusewitch GA, Chritton DB, Folz JC, Lebeck LK & Nehlsen-Cannarella SL (1993) Rapid flow cytometric assay for the assessment of natural killer cell activity. *J Immunol Methods* **166**, 45–54.
- Chapman MA (2001) The role of the colonic flora in maintaining a healthy large bowel mucosa. *Ann R Coll Surg Engl* **83**, 75–80.
- Chiang BL, Sheih YH, Wang LH, Liao CK & Gill HS (2000) Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. *Eur J Clin Nutr* **54**, 849–855.
- Christensen HR, Nexmann Larson C, Kæstel P, Buus Rosholm L, Sternberg C, Fleischer Michaelsen K & Frøkiær H (2006) Immunomodulating potential of supplementation with probiotics: a dose-response study in healthy young adults. *FEMS Immunol Med Microbiol* **47**, 380–390.
- Cross ML (2002) Microbes versus microbes: immune signals generated by probiotic lactobacilli and their role in protection against microbial pathogens. *FEMS Immunol Med Microbiol* **34**, 245–253.
- Delzenne N, Cherbut C & Neyrinck A (2003) Prebiotics: actual and potential effects in inflammatory and malignant colonic diseases. *Curr Opin Clin Nutr Metab Care* **6**, 581–586.
- Derynck R & Choy L (1998) Transforming growth factor-beta and its receptors. In *The Cytokine Handbook*, pp. 593–636 [AW Thomson, editor]. London: Academic Press.
- Erickson KL & Hubbard NE (2000) Probiotic immunomodulation in health and disease. *J Nutr* **130**, Suppl., 403S–409S.
- Femia AP, Luceri C, Dolara P, Giannini A, Biggeri A, Salvadori M, Clune Y, Collins KJ, Paglierani M & Caderni G (2002) Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* on azoxymethane-induced colon carcinogenesis in rats. *Carcinogenesis* **23**, 1953–1960.
- Flamm G, Glinsmann W, Kritchevsky D, Prosky L & Roberfroid M (2001) Inulin and oligofructose as dietary fiber: a review of the evidence. *Crit Rev Food Sci Nutr* **41**, 353–362.
- Fujiwara S, Akiyama M, Yamakido M, Seyama T, Kobuke K, Hakoda M, Kyoizumi S & Jones SL (1986) Cryopreservation of human lymphocytes for assessment of lymphocyte subsets and natural killer cytotoxicity. *J Immunol Methods* **90**, 265–273.
- Furrie E, MacFarlane S, Kennedy A, Cummings JH, Walsh SV, O'Neil DA & MacFarlane GT (2005) Synbiotic therapy (*Bifidobacterium longum*/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial. *Gut* **54**, 242–249.
- Gabrilovich D & Pisarev V (2003) Tumor escape from immune response: mechanisms and targets of activity. *Curr Drug Targets* **4**, 525–536.
- Gibson GR & Roberfroid M (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* **125**, 1401–1412.
- Gill HS & Cross ML (2002) Probiotics and immune function. In *Nutrition and Immune Function*, pp. 251–272 [PC Calder, CJ Field and HS Gill, editors]. Wallingford, UK: CABI International.
- Gill HS, Rutherford KJ, Cross ML & Gopal PK (2001) Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN0191. *Am J Clin Nutr* **74**, 833–839.
- Guigoz Y, Rochat F, Perruisseau-Carrier G, Rochat I & Schiffrin EJ (2002) Effects of oligosaccharide on the faecal flora and non-specific immune system in elderly people. *Nutr Res* **22**, 13–25.
- Hacker G, Redecke V & Hacker H (2002) Activation of the immune system by bacterial CpG-DNA. *Immunology* **105**, 245–251.
- Halpern GM, Vruwink KG, Vandewater J, Keen CL & Gershwin ME (1991) Influence of long-term yoghurt consumption in young-adults. *Int J Immunother* **7**, 205–210.
- Harris SG, Padilla J, Koumas L, Ray D & Phipps RP (2002) Prostaglandins as modulators of immunity. *Trends Immunol* **23**, 144–150.
- Kishi A, Uno K, Matsubara Y, Okuda C & Kishida T (1996) Effect of the oral administration of *Lactobacillus brevis* subsp. *coagulans* on interferon-alpha producing capacity in humans. *J Am Coll Nutr* **15**, 408–412.
- Kvale D & Brandtzaeg P (1995) Constitutive and cytokine induced expression of HLA molecules, secretory component, and intercellular adhesion molecule-1 is modulated by butyrate in the colonic epithelial cell line HT-29. *Gut* **36**, 737–742.
- Lindsay JO, Whelan K, Stagg AJ, Gobin P, Al-Hassi HO, Rayment N, Kamm MA, Knight SC & Forbes A (2006) Clinical, microbiological, and immunological effects of fructo-oligosaccharide in patients with Crohn's disease. *Gut* **55**, 348–355.
- Marteau P, Seksik P & Jian R (2002) Probiotics and health: new facts and ideas. *Curr Opin Biotechnol* **13**, 486–489.
- Meydani SN & Ha WK (2000) Immunologic effects of yogurt. *Am J Clin Nutr* **71**, 861–872.
- Nagao F, Nakayama M, Muto T & Okumura K (2000) Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the immune system in healthy human subjects. *Biosci Biotechnol Biochem* **64**, 2706–2708.
- O'Gorman MR (2002) Evaluation of phagocytic cell function. In *Manual of Clinical Laboratory Immunology*, pp. 265–283 [NR Rose, RG Hamilton and B Detrick, editors]. Washington, DC: ASM Press.



- Olivares M, Diaz-Ropero MP, Gomez N, Lara-Villoslada F, Sierra S, Maldonado JA, Martin R, Rodriguez JM & Xaus J (2006) The consumption of two new probiotic strains *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711, boosts the immune system of healthy humans. *Int Microbiol* **9**, 47–52.
- Ouwehand AC, Isolauri E, Kirjavainen PV, Tolkkio S & Salminen SJ (2000) The mucus binding of *Bifidobacterium lactis* Bb12 is enhanced in the presence of *Lactobacillus* GG and *Lact. delbrueckii* subsp *bulgaricus*. *Lett Appl Microbiol* **30**, 10–13.
- Pasche B (2001) Role of transforming growth factor beta in cancer. *J Cell Physiol* **186**, 153–168.
- Perdigon GFR & Raya R (2002) Lactic acid bacteria and their effect on the immune system. *Curr Issues Intest Microbiol* **2**, 27–42.
- Pugh S & Thomas GAO (1994) Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E(2). *Gut* **35**, 675–678.
- Rafter J (2003) Probiotics and colon cancer. *Best Pract Res Clin Gastroenterol* **17**, 849–859.
- Rhind SG, Gannon GA, Suzui M, Shephard RJ & Shek PN (1999) Indomethacin inhibits circulating PGE2 and reverses postexercise suppression of natural killer cell activity. *Am J Physiol* **276**, R1496–R1505.
- Rigas B, Goldman IS & Levine L (1993) Altered eicosanoid levels in human colon-cancer. *J Lab Clin Med* **122**, 518–523.
- Roller M, Femia AP, Caderni G, Rechkemmer G & Watzl B (2004a) Intestinal immunity of rats with colon cancer is modulated by oligofructose-enriched inulin combined with *Lactobacillus rhamnosus* and *Bifidobacterium lactis*. *Br J Nutr* **92**, 931–938.
- Roller M, Rechkemmer G & Watzl B (2004b) Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats. *J Nutr* **134**, 153–156.
- Sanderson IR (2004) Short chain fatty acid regulation of signaling genes expressed by the intestinal epithelium. *J Nutr* **134**, suppl., 2450S–2454S.
- Schley PD & Field CJ (2002) The immune-enhancing effects of dietary fibres and prebiotics. *Br J Nutr* **87**, S221–S230.
- Sheih YH, Chiang BL, Wang LH, Liao CK & Gill HS (2001) Systemic immunity-enhancing effects in healthy subjects following dietary consumption of the lactic acid bacterium *Lactobacillus rhamnosus* HN001. *J Am Coll Nutr* **20**, suppl., 149–156.
- Smyth MJ, Godfrey DI & Trapani JA (2001) A fresh look at tumor immunosurveillance and immunotherapy. *Nature Immunol* **2**, 293–299.
- Spanhaak S, Havenaar R & Schaafsma G (1998) The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. *Eur J Clin Nutr* **52**, 899–907.
- van Loo J, Clune Y, Bennett M & Collins JK (2005) The SYNCAN project: goals, set-up, first results and settings of the human intervention study. *Br J Nutr* **93**, Suppl. 1, S91–S98.
- Watzl B, Gırrbach S & Roller M (2005) Inulin, oligofructose and immunomodulation. *Br J Nutr* **93**, Suppl. 1, S49–S55.
- Yakar I, Melamed R, Shakhar G, Shakhar K, Rosenne E, Abudarham N, Page GG & Ben-Eliyahu S (2003) Prostaglandin e(2) suppresses NK activity *in vivo* and promotes postoperative tumor metastasis in rats. *Ann Surg Oncol* **10**, 469–479.