Nutritional Immunology—Research Communication

Prebiotic Inulin Enriched with Oligofructose in Combination with the Probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* Modulates Intestinal Immune Functions in Rats[1]

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ABSTRACT   Probiotics (PRO) modulate systemic immunity in animals and humans. In contrast, the effects of prebiotics (PRE) on systemic and intestinal immunity have not been investigated. Whether the combined application of PRO and PRE [synbiotics (SYN)] has synergistic or additive effects is presently unknown. Therefore, PRO (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12), PRE (inulin enriched with oligofructose), and SYN (combination of PRO and PRE) were fed to F344 rats for 4 wk as supplements to a high fat diet. Functions of immune cells isolated from peripheral blood mononuclear cells (PBMC), spleen, mesenteric lymph nodes and Peyer's patches (PP) were investigated. The SYN supplement increased secretory immunoglobulin A (sIgA) production in the ileum compared with controls fed the high fat diet alone ($P < 0.05$), and decreased the oxidative burst activity of blood neutrophils ($P < 0.05$) compared with rats fed PRO. The PRE supplement enhanced the production of interleukin-10 ($P < 0.05$) in PP as well as the production of sIgA in the cecum ($P < 0.05$), compared with controls. The PRO supplement modestly affected immune functions, whereas systemic immunomodulatory effects were observed in rats fed SYN. The PRE supplement primarily acted at the level of the gut-associated lymphoid tissue. The combined application of PRO and PRE has different effects from those of the individual supplements, but does not simply result in additive or synergistic effects.   J. Nutr. 134: 153–156, 2004.

KEY WORDS: • Immune sytem • gut • probiotic • prebiotic • synbiotic • rat

Several recent reviews summarized the available experimental evidence for the immunomodulatory effects of probiotics (PRO)[4] (1,2). Although experimental and human studies clearly show that PRO affect host resistance to intestinal infections as well as a number of immune cell functions, the results of these studies vary greatly. In addition, the underlying immunological mechanisms of PRO are generally not well defined (3,4). The gut-associated lymphoid tissue (GALT) is increasingly recognized as a crucial component of immune response (5). Although many studies have focused on the systemic immunological effects of PRO, few data are available regarding systemic immunological effects and local immunological effects in the gut in the same animal (6). Therefore, studies investigating the immunomodulatory potential of PRO, including their effects at the GALT level, are needed.

At present, few studies have investigated the direct effects of prebiotics (PRE) on the immune system (7). Whether PRE modulate the immune response directly or indirectly, by affecting the composition of the intestinal flora and thus affecting the GALT, or by producing SCFA, is presently unknown.

Our study investigated the effects of PRO, PRE and their combined application [synbiotics (SYN)] at the systemic level and at the GALT level. We hypothesized that PRO and PRE, alone or in combination, would modify immune responses in the GALT, enhancing cytokine production and other immune cell functions. Therefore, we fed F344 rats consuming a high fat, low fiber control diet a daily supplement consisting of two probiotic strains (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12), an inulin-based prebiotic enriched with oligofructose or the combination of PRO and PRE (SYN) for a period of 4 wk. We decided to use two probiotic strains because others have suggested that a mixture of PRO may have a greater effect on the intestine than the individual strains (8,9). We assessed a broad spectrum of immune functions with cells isolated from peripheral blood mononuclear cells (PBMC) and the spleen as well as from the GALT. We used a Western-style high fat, low fiber diet instead of a standard rat diet as the control diet to make the study conditions more comparable to the situation of humans in Western countries consuming PRO and/or PRE.

MATERIALS AND METHODS

Animals. The State Veterinary Office granted permission for the rat studies, and the experiments complied with its guidelines for the care and use of laboratory animals. Male Fischer 344/NHsd rats (Harlan Winkelmann, Borchen, Germany) aged 12 to 13 wk were fed a standard lab diet (Altromin, Lage, Germany) for 1 wk, and all rats were then fed the control diet for 1 wk.

Diets. Dietary components were purchased from Piccioni (Gessate, Milan, Italy). RaftiloseR Synergy1 was provided by Orafti (Tienen, Belgium). This PRE is an oligofructose-enriched inulin,

[4] Abbreviations used: Bb12, *Bifidobacterium lactis* Bb12; BSA, bovine serum albumin; CFU, colony-forming units; ConA, concanavalin A; GALT, gut-associated lymphoid tissue; HF, high fat; LAB, lactic acid bacteria; LGG, *Lactobacillus rhamnosus* GG; MLN, mesenteric lymph nodes; NK, natural killer; PBMC, peripheral blood mononuclear cells; PP, Peyer's patches; PRE, prebiotics; PRO, probiotics; PTX, pentoxifylline; sIgA, secretory immunoglobulin A; SYN, synbiotic.

comprised of a 1:1 mixture of long- and short-chain fractions of inulin, a $\beta(2-1)$-fructan extracted from chicory roots (*Cichorium intybus*). *L. rhamnosus* GG (LGG) and *B. lactis* Bb12 (Bb12) were provided by Valio (Helsinki, Finland) and purchased from Chr. Hansen (Horsholm, Denmark), respectively. They were supplied as freeze-dried powder in sealed packets containing $\sim 6 \times 10^{11}$ colony-forming units (CFU)/g (LGG) and $\sim 3 \times 10^{10}$ CFU/g (Bb12), respectively. The bacteria were stored at a temperature of $-20°C$ until used. The dietary concentration of LGG and Bb12 was evaluated as described elsewhere (10).

Rats ($n = 80$) were allocated to four experimental groups ($n = 20$). The control group was fed a high fat (HF) diet based on the AIN76 diet (11), modified to contain a high level of fat (231 g corn oil/kg) and a low level of cellulose (20 g/kg) to compare with the diet typical of Western humans at high risk of colon cancer (10). The sources of carbohydrates in this diet were sucrose (361 g/kg) and maltodextrins (100 g/kg). The PRO group was fed an HF diet supplemented with LGG and Bb12 to provide $\sim 5 \times 10^{11}$ CFU of each strain per kg of diet. The PRE group was fed an HF diet with the maltodextrins replaced by 100 g/kg of Raftilose Synergy1. The SYN group was fed the PRE-group diet supplemented with LGG and Bb12 to provide $\sim 5 \times 10^{11}$ CFU of each strain per kg of diet, matching the levels in the PRO-group diet. Food and water were consumed ad libitum. Diets were prepared every week, divided into aliquots and frozen at $-20°C$. At the end of the 4-wk experimental feeding period, the rats were anesthetized with CO_2 and then decapitated.

Preparation of immune cell suspensions and fecal samples. Immune cell suspensions were prepared with RPMI-1640 culture medium containing 50 mL/L of heat-inactivated fetal bovine serum, L-glutamine (2 mmol/L), penicillin (1×10^5 U/L), streptomycin (100 mg/L) and HEPES (25 mmol/L); all components were purchased from Life Sciences (Eggenstein-Leopoldshafen, Germany). Trunk blood was collected in heparinized tubes after decapitation. The PBMC and splenocytes were isolated as previously described (12). The intestinal contents of the ileum and cecum were collected and stored at $-20°C$ until assayed.

Fluorescence staining of lymphocyte subpopulations. The expression of cell surface markers on the immune cells of blood, spleen and mesenteric lymph nodes (MLN) was investigated by immunofluorescence as previously described (12). Phycoerythrin-conjugated mouse anti-rat monoclonal antibodies to CD4 (Caltag, Hamburg, Germany) and fluorescein-conjugated mouse anti-rat monoclonal antibodies to CD8 (Caltag) with appropriate isotype controls (Caltag) were used. Samples were analyzed with a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany).

Phagocytosis. The phagocytic capacity of the immune cells of the blood and the spleen was assessed with a flow cytometric method as previously described (13).

Oxidative burst. Whole blood (100 μL) was cooled on ice to 0°C. Tubes were incubated with 20 μL PBS (control) or 20 μL phorbol myristate 13-acetate (8.1 μmol/L; Sigma-Aldrich, Deisenhofen, Germany), as a high stimulus for 10 min at 37°C. Dihydrorhodamine 123 (1.7 μL, 29 mmol/L; Molecular Probes, Leiden, Netherlands) was added and samples were incubated for 15 min at 37°C. Erythrocytes were lysed with 2 mL 1× lysing solution. After centrifugation (250 × g, 4°C, 5 min), cells were washed with 3 mL PBS. The cells were resuspended in 300 μL propidium iodide (0.75 μmol/L; Sigma-Aldrich) and incubated for 10 min on ice. Samples were analyzed within 30 min with the flow cytometer.

Cytotoxicity of natural killer (NK) cells. The natural killer (NK) cell activity of the immune cells of the blood, spleen, MLN and PP was assessed with a flow cytometric method as previously described (14), using target cells from the mouse Moloney leukemia cell line YAC-1.

Lymphocyte proliferation. The ex vivo proliferative responsiveness of lymphocytes isolated from the spleen, MLN (each 5×10^9 cells/L) and PP (7×10^9 cells/L) to the mitogen concanavalin A (ConA; 1 mg/L; Sigma-Aldrich, Deisenhofen, Germany) for 72 h at 37°C, 5% CO_2 and 95% humidity was determined by ELISA, using a commercial proliferation kit (Cell Proliferation ELISA kit; Roche Diagnostics, Mannheim, Germany).

Cytokines. Splenocytes (100 μL; 1×10^9 cells/L IFN-γ and 5×10^9 cells/L IL-10), MLN (100 μL; 1×10^9 cells/L IFN-γ and 5×10^9 cells/L IL-10) and PP (100 μL; 5×10^9 cells/L IFN-γ and 5×10^9 cells/L IL-10) were stimulated by 100 μL of Con A (5 mg/L) for 24 h at 37°C, 5% CO_2 and 95% humidity. Levels of IFN-γ and IL-10 in the supernatants were measured with OptEIA commercial ELISA kits (BD Pharmingen, Heidelberg, Germany), following the manufacturers' instructions.

Secretory immunoglobulin A (sIgA). Fresh caecal (140 mg/rat) and ileal contents (10 mg/rat) were mixed with 1 mL of a solution (10 g/L) of bovine serum albumin (BSA; Sigma-Aldrich) in PBS and incubated for 10 min at room temperature. Samples were centrifuged (4000 × g, 30 min, 20°C) and supernatants were collected and stored at $-20°C$ until assayed. Maxisorb 96-well microtiter plates (NUNC, Roskilde, Denmark) were coated with 100 μL of a rabbit anti-rat secretory component antibody (Bethyl Laboratories, Montgomery, AL) diluted 1:1000 with a solution (50 g/L) of Tween 20 (Sigma) in PBS and incubated overnight at 4°C. After blocking with 100 μL of BSA (40 g/L) in the Tween solution for 1 h and washing, 100-μL titers of the samples were applied. After incubation for 2 h, 100 μL of horseradish peroxidase-conjugated goat monoclonal anti-rat IgA (Bethyl Laboratories) diluted 1:500 with the Tween-BSA solution was added, and the plates were then incubated for 2 h. A peroxidase substrate, 100 μL of tetramethylbenzidine (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was then added, and the plates were incubated for 30 min in the dark. The enzyme reaction was stopped with 100 μL of 1 mol/L H_3PO_4. The relative amount of secretory immunoglobulin A (sIgA) was quantified by measuring the absorbance at 450 nm.

Statistics. Results are reported as means ± SD. Differences among groups were tested for significance by one-way ANOVA with the Tukey-Kramer test for comparison of individual means when appropriate. To assess correlations between single immunological markers, Pearson correlation coefficients were computed. Values of $P < 0.05$ were considered significant. All statistical calculations were performed with the StatView program (1998 release, SAS Institute, Cary, NC).

RESULTS

Supplementation of the control diet with PRO, PRE and SYN for 4 wk did not affect feed intake (15.0 ± 1.05 g/d), weight gain (46 ± 11 g), and final body weight (370 ± 21 g). The intake of LGG was $\sim 7.3 \pm 0.5 \times 10^9$ CFU/d, and intake of Bb12 was $7.6 \pm 0.6 \times 10^9$ CFU/d.

No significant differences in $CD4^+$ and $CD8^+$ T-lymphocytes were observed among the treatment groups in any of the tissues studied (data not shown). In the blood the CD4:CD8 ratio tended to be greater ($P = 0.08$) in rats treated with PRO or SYN than in the control or PRE groups (control, 1.80 ± 0.22; PRE, 1.81 ± 0.16; PRO, 1.95 ± 0.35; SYN, 1.95 ± 0.21).

Neutrophil and monocyte phagocytosis (percentage active cells and mean fluorescence intensity) were not affected by PRO, PRE or SYN (data not shown). Oxidative burst activity was significantly reduced in blood neutrophils isolated from rats treated with SYN compared with rats treated with PRO (**Table 1**).

The treatments tended ($P = 0.09$) to affect NK cell activity in PBMC (control, 15 ± 6%; PRE, 16 ± 5%; PRO, 18 ± 6%; SYN, 19 ± 6%). The NK cell activity did not differ among the groups in any other tissue studied. Lymphocyte proliferation was not affected by the dietary treatments in any of the tissues studied (data not shown).

The treatments did not modulate cytokine production in the spleen or MLN (data not shown), but PRE treatment increased IL-10 production in PP relative to the control group ($P < 0.05$; **Table 2**). The production of IFN and IL-10 in this tissue were correlated ($r = 0.90$; $P < 0.0001$).

TABLE 1

Oxidative burst activity in peripheral blood neutrophils of rats fed a high fat diet supplemented with a probiotic (PRO), a prebiotic (PRE) and a synbiotic (SYN)[1]

Parameter	Control	PRO	PRE	SYN
Active cells, %	41 ± 14	40 ± 13	40 ± 10	38 ± 11
Fluorescence intensity	47 ± 5ab	48 ± 7a	44 ± 6ab	42 ± 6b

[1] Values are means ± SD, $n = 20$. Means in a row without a common letter differ, $P < 0.05$.

The SYN treatment enhanced the sIgA concentration in the ileum ($P < 0.05$), and the PRE treatment increased it in the cecum ($P < 0.05$; **Table 3**), relative to the control group.

DISCUSSION

The objectives of the present study were to investigate whether the effects of PRO and PRE on the immune system vary among different immune compartments and whether SYN has a greater effect than that obtained with PRO or PRE supplementation alone. The results demonstrate that PBMC and PP are the primary tissues that are specifically affected by PRE. In addition, although PRE supplementation alone induced significant immunomodulation in the intestine, PRO supplementation was primarily effective when provided as a component of SYN.

We used a high fat, low-fiber diet instead of a standard rat diet because it more closely resembles the dietary conditions of Western humans consuming PRO and/or PRE. The fat content of the diet affects immune functions such as NK cell activity (15). Therefore, the potential immunomodulatory effects of PRO and PRE may become more obvious in subjects fed such a diet.

Overall, the treatments did not affect the subpopulations of lymphocytes in the blood, spleen and MLN. However, the PRO and SYN treatments tended to increase the CD4:CD8 ratio in the blood. This suggests that LGG + Bb12 treatment modulates the composition of circulating lymphocytes in the periphery. In another study with rats, consuming yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) for 4 wk did not affect lymphocyte subpopulations in the PBMC and spleen when compared with unfermented milk (6). In mice, consuming lactic acid bacteria (LAB) for 4 wk did not affect the percentages of CD4$^+$ and CD8$^+$ cells in the blood (16).

TABLE 2

Cytokine production of Peyer's patch (PP) cells of rats fed a high fat diet supplemented with a probiotic (PRO), a prebiotic (PRE) and a synbiotic (SYN)[1,2]

Cytokine	Control	PRO	PRE	SYN
		μg/L		
IFN	2.7 ± 0.8	2.7 ± 1.0	3.6 ± 2.0	2.9 ± 1.2
IL-10	0.31 ± 0.13a	0.37 ± 0.19ab	0.56 ± 0.38b	0.46 ± 0.29ab

[1] Values are means ± SD, $n = 20$. Means in a row without a common letter differ, $P < 5$.
[2] Lymphocytes were stimulated with 5 mg/L concanavalin A (ConA) and cultivated for 24 h.

TABLE 3

Secretory immunoglobulin A (sIgA) concentrations in ileum and cecum of rats fed a high fat diet supplemented with a probiotic (PRO), a prebiotic (PRE) and a synbiotic (SYN)[1]

Tissue	Control	PRO	PRE	SYN
		OD_{450}		
Ileum	0.78 ± 0.36a	0.92 ± 0.41ab	1.09 ± 0.62ab	1.30 ± 0.75b
Caecum	0.66 ± 0.42a	0.88 ± 0.59ab	1.15 ± 0.77b	0.72 ± 0.31ab

[1] Values are means ± SD, $n = 20$. Means in a row without a common letter differ, $P < 0.05$.

Altogether these data suggest that PRO and PRE have only minor effects on the composition of T cell subsets in different immune compartments.

In contrast to a number of animal and human studies (17), the present study found that PRO treatment did not stimulate neutrophil and monocyte phagocytosis. A study with humans also found that *Lacobacillus casei* Shirota supplementation for 4 wk did not affect phagocytosis in healthy adults (18). Because the subjects of that study also consumed a high fat diet comparable to the one used in the present study with rats, the basic diet might be at least partly responsible for the observed differences. The reason for the significant reduction of the oxidative burst activity in the SYN group is unknown.

In the present study, lymphocytes from the blood, spleen and GALT, including PP and MLN, exerted an NK-like cytotoxic effect (based on their capacity to lyse the YAC-1 target cell line). Because no earlier study reported NK cell activity in rat PP, we used pentoxifylline (PTX), a suppressor of NK cell activity (19), to suppress NK cell activity in the spleen and PP. The PTX suppressed NK cell activity in both tissues (data not shown).

The PRO and SYN treatments tended to enhance NK cell activity only in blood. This suggests that LGG + Bb12 supplementation may have caused this increase. Studies in humans have demonstrated that *L. rhamnosus* (strain HNOO1) and *B. lactis* (strain HN019) supplementation upregulate PBMC NK cell cytotoxicity (17), although the overall nutritional status of the study subjects also modulated the effect of this PRO treatment (20). These data support the observations of the present study with rats. Supplementation with *B. lactis* in oligosaccharide-enriched milk caused higher NK cell cytotoxicity in humans than the PRO treatment without the oligosaccharide (21).

The capacity of lymphocytes isolated from the spleen, MLN and PP to proliferate following mitogen activation was not affected by the dietary treatments. This conflicts with animal studies on strains of LAB which found that consuming yogurt ($\sim 1.4 \times 10^9$ bacteria/d) or pure bacteria increased the proliferative responsiveness of lymphocytes to ConA (6,16). Again, differences in the composition of the control diets may explain the differing outcomes of these studies.

The PRE treatment significantly stimulated IL-10 production by PP cells. In PP, IFN and IL-10 are primarily produced by T-helper1 lymphocytes (IFN) and by T-helper2/T-regulatory lymphocytes and dendritic cells (IL-10). The strong correlation between the level of production of the two cytokines ($r = 0.90$, $P < 0.0001$) suggests that the PRE treatment simultaneously activated different T-lymphocyte subpopulations and/or dendritic cells. The PRE treatment did not affect splenocytes or mesenteric lymphocytes, which constitutively

express lower levels of multiple cytokine transcripts than PP (22). In contrast to the strong effects of PRE on PP cytokine production, the combined treatment with PRO and PRE abrogated this effect. Because PRE did not affect the proliferative responsiveness of PP to the mitogen ConA, the increase in cytokine production indicates that PRE and/or its metabolites may interfere specifically with the regulatory processes of cytokine production. A recent study with mice also reported that consuming fructooligosaccharide enhanced IFN and IL-10 production by PP cells (23), confirming the results of the present study. Consuming the prebiotic raffinose further increased IL-12 production of PP (24), which enhances IFN production in PP (25). However, feeding mice yogurt LAB (*L. bulgaricus, S. thermophilus*) had no effect on basal IFN mRNA expression in PP (26). In another study, feeding mice *L. rhamnosus* for 4 wk significantly increased IFN production by splenocytes, but *B. lactis* had no effect (18). Because we used both LAB strains in combination, this may have impeded enhanced IFN production by splenocytes in the present study.

Treatment with SYN (ileum) and PRE (cecum) increased concentrations of total sIgA in rats, whereas PRO alone had no effect. The availability of PRE in the ileum may have supported the growth of the supplemented PRO and consequently stimulated sIgA synthesis. In contrast, in the cecum there is a large quantity of endogenous microorganisms; PRE supported the growth of these bacteria, which also stimulated sIgA production. However, in rats treated with SYN, the PRE may have been metabolized in the ileum and therefore could not further support bacterial growth in the cecum. Our results are in line with those of a recent study that found that feeding mice fructooligosaccharides increased fecal IgA concentration (23). The mechanism of this sIgA-enhancing effect is unknown. The IFN stimulates expression of the secretory component for IgA by epithelial cells (27). However, we found no correlation between changes in IFN production by PP and caecal sIgA concentration with the PRE treatment (data not shown). This argues against a potential role of IFN.

In conclusion, PBMC are sensitive (CD4$^+$, oxidative burst, NK) to PRO and SYN treatment, whereas PP are primarily sensitive to PRE treatment (cytokine and sIgA production). The spleen and MLN were not affected by any of the treatments. The present data suggest that PRO and PRE act via different mechanisms. Because we used a high fat, low fiber diet rather than the standard low fat rat diet used by most other studies, our results may be more relevant to the situation of humans consuming PRO or PRE in Western-style diets.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Erickson, K. L. & Hubbard, N. E. (2000) Probiotic immunomodulation in health and disease. J. Nutr. 130: 403S–409S.

2. Meydani, S. N. & Ha, W. K. (2000) Immunologic effects of yoghurt. Am. J. Clin. Nutr. 71: 861–872.

3. Cross, M. L. (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.

4. Teitelbaum, J. E. & Walker, W. A. (2002) Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. Annu. Rev. Nutr. 22: 107–138.

5. Mowat, A. M. (2003) Anatomical basis of tolerance and immunity to intestinal antigens. Nature Rev. Immunol. 3: 331–341.

6. Aattouri, N., Bouras, M., Tome, D., Marcos, A. & Lemonnier, D. (2002) Oral ingestion of lactic-acid bacteria by rats increases lymphocyte proliferation and interferon-γ production. Brit. J. Nutr. 87: 367–373.

7. Schley, P. D. & Field, C. J. (2002) The immuno-enhancing effects of dietary fibres and prebiotics. Brit. J. Nutr. 87(suppl. 2): S221–S230.

8. Campieri, M. & Giochetti, P. (1999) Probiotics in inflammatory bowel disease: new insight to pathogenesis or a possible therapeutic alternative? Gastroenterology 116: 1246–1250.

9. Ouwehand, A. C., Isolauri, E., Kirjavainen, P. V., Tolkko, S. & Salminen, S. J. (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.

10. Femia, A. P., Luceri, C., Dolara, P., Giannini A., Biggeri, A., Salvadori, M., Clune Y., Collins, K. J., 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.

11. American Institute of Nutrition (1977) Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J. Nutr. 107: 1340–1348.

12. Watzl, B., Neudecker, C., Hänsch, G. M., Rechkemmer, G. & Pool-Zobel, B. L. (1999) Short-term moderate aflatoxin B$_1$ exposure has only minor effects on the gut-associated lymphoid tissue of Brown Norway rats. Toxicology 138: 93–102.

13. Watzl, B., Bub, A., Briviba, K. & Rechkemmer, G. (2002) Acute intake of moderate amounts of red wine or alcohol has no effect on the immune system of healthy men. Eur. J. Nutr. 41: 264–270.

14. Watzl, B., Bub, A., Blockhaus, M., Herbert, B. M., Lührmann, P. M., Neuhäuser-Berthold, M. & Rechkemmer, G. (2000) Prolonged tomato juice consumption has no effect on cell-mediated immunity of well-nourished elderly men and women. J. Nutr. 130: 1719–1723.

15. Barone J., Hebert, J. R. & Reddy, M. M. (1989) Dietary fat and natural-killer-cell activity. Am. J. Clin. Nutr. 50: 861–867.

16. Gill, H. S., Rutherfurd, K. J., Prasad, J. & Gopal, P. K. (2000) Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus (HN001), Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). Brit. J. Nutr. 83: 167–176.

17. Gill, H. S. & Cross, M. L. (2002) Probiotics and immune function. In: Nutrition and Immune Function (Calder, P. C., Field, C. J. & Gill, H. S., eds.), pp. 251–272. CABI International, Wallingford, UK.

18. 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.

19. Reed, W. R. & DeGowin, R. L. (1992) Suppressive effects of pentoxifylline on natural killer cell activity. J. Lab. Clin. Med. 119: 763–771.

20. Gill, H. S., Rutherfurd, K. J. & Cross, M. L. (2001) Dietary probiotic supplementation enhances natural killer cell activity in the elderly: an investigation of age-related immunological changes. J. Clin. Immunol. 21: 264–271.

21. Chiang, B. L., Sheih, Y. H., Wang, L. H., Liao, C. K. & Gill, H. S. (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.

22. Lagoo, A. S., Eldridge, J. H., Lagoo-Deenadaylan, S., Black, C. A., Ridwan, B. U., Hardy, K. J., McGhee, J. R. & Beagley, K. W. (1994) Peyer's patch CD8$^+$ memory T cells secrete T helper type 1 and type 2 cytokines and provide help for immunoglobulin secretion. Eur. J. Immunol. 24: 3087–3092.

23. Hosono, A., Ozawa, A., Kato, R., Ohnishi, Y., Nakanishi, Y., Kimura, T. & Nakamura, R. (2003) Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. Biosci. Biotechnol. Biochem. 67: 758–764.

24. Nagura, T., Hachimura, S., Hashiguchi, M., Ueda, Y., Kanno, T., Kikuchi, H., Sayama, K. & Kaminogawa, S. (2002) Suppressive effect of dietary raffinose on T-helper 2 cell-mediated immunity. Brit. J. Nutr. 88: 421–426.

25. MacDonald, T. T. & Monteleone, G. (2001) IL-12 and Th1 immune responses in human Peyer's patches. Trends Immunol. 22: 244–247.

26. Tejada-Simon, M. V., Ustunol, Z. & Pestka, J. J. (1999) Effects of lactic acid bacteria ingestion on basal cytokine mRNA and immunoglobulin levels in the mouse. J. Food Protect. 62: 287–291.

27. Sollid, L. M., Kvale, D., Brandtzaeg, P., Markussen, G. & Thorsby, E. (1987) Interferon-gamma enhances expression of secretory component, the epithelial receptor for polymeric immunoglobulins. J. Immunol. 138: 4303–4306.