# Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019)

H. S. Gill[1,2]*, K. J. Rutherfurd[1,2], J. Prasad[2] and P. K. Gopal[2]

[1]*Milk and Health Research Centre, Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand*

[2]*New Zealand Dairy Research Institute, Palmerston North, New Zealand*

*(Received 1 February 1999 – Revised 2 August 1999 – Accepted 16 August 1999)*

Consumption of lactic acid bacteria (LAB) has been suggested to confer a range of health benefits including stimulation of the immune system and increased resistance to malignancy and infectious illness. In the present study, the effects of feeding *Lactobacillus rhamnosus* (HN001, DR20™), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019, DR10™) on *in vivo* and *in vitro* indices of natural and acquired immunity in healthy mice were examined. Mice were fed daily with *L. rhamnosus*, *L. acidophilus* or *B. lactis* ($10^9$ colony forming units) and their immune function was assessed on day 10 or day 28. Supplementation with *L. rhamnosus*, *L. acidophilus* or *B. lactis* resulted in a significant increase in the phagocytic activity of peripheral blood leucocytes and peritoneal macrophages compared with the control mice. The proliferative responses of spleen cells to concanavalin A (a T-cell mitogen) and lipopolysaccharide (a B-cell mitogen) were also significantly enhanced in mice given different LAB. Spleen cells from mice given *L. rhamnosus*, *L. acidophilus* or *B. lactis* also produced significantly higher amounts of interferon-γ in response to stimulation with concanavalin A than cells from the control mice. LAB feeding had no significant effect on interleukin-4 production by spleen cells or on the percentages of $CD4^+$, $CD8^+$ and $CD40^+$ cells in the blood. The serum antibody responses to orally and systemically administered antigens were also significantly enhanced by supplementation with *L. rhamnosus*, *L. acidophilus* or *B. lactis*. Together, these results suggest that supplementation of the diet with *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) is able to enhance several indices of natural and acquired immunity in healthy mice.

**Probiotics: Immunity: Lactic acid bacteria**

Deficiencies in the functioning of the immune system as a consequence of old age, stress, infectious illness (e.g. acquired immune deficiency syndrome) and malnutrition are well documented (Edelman & Zolla-Pazner, 1989; Khansari *et al*. 1990; Goodwin, 1995; Pawelec *et al*. 1995; Woodward, 1998). The findings that these immunological deficiencies could be overcome by modulating the immune system have stimulated a search for natural and chemical agents and/or products with immunomodulatory properties. Unfortunately, the use of many currently available immunostimulatory products is associated with deleterious side-effects (Bardana, 1985; Nussler & Thomson, 1992). The development of natural food products with immunoenhancing properties, that are free of side-effects, would therefore be of significant benefit to population groups with impaired immune function.

Lactobacilli and bifidobacteria are normal components of the healthy human intestinal microflora and are commonly used for fermentation of food products. Recent studies have shown that consumption of some lactobacilli and/or bifidobacteria is able to confer a range of health benefits including enhancement of immunity and improved resistance to infectious illness and cancers (reviewed by Gill, 1998; Goldin, 1998). Immune stimulation has been suggested to underlie anti-infection and anti-carcinogenic effects of lactic acid bacteria (LAB). These findings have caught the attention of nutrition, health and food scientists and have stimulated interest in finding new strains of lactobacilli and

**Abbreviations:** ConA, concanavalin A; CT, cholera toxin; IFN-γ, interferon-γ; IgE, immunoglobulin E; IL, interleukin; LAB, lactic acid bacteria; NK, natural killer; PBL, peripheral blood leucocytes; TT, tetanus toxoid.

***Corresponding author:** Professor H. S. Gill, fax +64 6 350 5446, email h.s.gill@massey.ac.nz

bifidobacteria that are able to enhance immunity. It is important to note, however, that a large variation exists in the ability of LAB strains to enhance immune function and only a few strains with well-established immunity-enhancing effects have been identified to date (Gill, 1998; Salminen *et al.* 1998). Also, there are only a few published studies where the effects of LAB on a broad spectrum of host immune responses have been studied. It is well established that different components of the immune system act in concert to mediate protection and that the most successful host immune responses involve the activation of both natural and acquired (antibody and cell-mediated) immune responses (Roitt *et al.* 1989; Zinkernagal & Hengartner, 1997). Therefore, the strains of lactobacilli or bifidobacteria that are able to affect a wider array of immune functions are likely to be more beneficial for human health.

The objective of the present study was to examine the effect of feeding *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) on various indices of immune function in healthy mice. The results of this study demonstrate that supplementation of the diet with *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) is able to enhance both natural and acquired immunity in mice.

## Materials and methods

### *Animals*

Male 6–7-week-old BALB/c mice bred and raised (under conventional conditions) at the Small Animal Production Unit, Massey University, were used. They were housed in individual cages at $22 \pm 1°$ under a 12 h light–dark photoperiod and offered feed and water *ad libitum*.

### *Lactic acid bacteria*

*L. rhamnosus* strain HN001 (DR20™), *L. acidophilus* strain HN017 and *B. lactis* strain HN019 (DR10™) isolated from dairy products were used. These strains were selected from a collection of over 2500 LAB strains held at the New Zealand Dairy Research Institute for their superior ability to adhere to human intestinal epithelial cells, and resist the effects of low pH and bile salts *in vitro* (Prasad *et al*. 1999). For feeding studies, bacteria were grown overnight at 37° in de Mann, Rogasa, Sharpe (MRS) or MRSC (MRS containing 0·5 g cysteine hydrochloride/l) broth and enumerated using MRS agar plates.

### *Experimental design*

Two experiments were conducted. In the first experiment, eighty mice were randomly allocated to four groups, and offered a milk-based diet (g/kg: skimmed milk powder (380 g protein/kg) 526, mineral mix 50, vitamin mix 50, cellulose 10, maize flour 284, maize oil (fat) 80) and water *ad libitum*. After acclimatization for 1 week, mice were orally administered with $10^9$ colony-forming units (CFU)/d *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) in 50 μl skimmed milk (100 g/l) for 10 d. Control mice received 50 μl skimmed milk without LAB. Mice were immunized orally with cholera toxin (10 μg in 25 μl 0·1 M-$NaH_2CO_3$; Sigma, St Louis, MO, USA) on days 0 and 7. Peritoneal macrophages, blood, spleens and intestinal washings were obtained on day 10 after humanely killing the mice.

In the second experiment, seventy-two mice were randomly allocated to four groups and offered feed (milk-based diet) and water *ad libitum*. After acclimatization, mice were orally administered with $10^9$ CFU/d *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) in 50 μl skimmed milk (100 g/l) for 28 d. Mice were immunized subcutaneously with tetanus vaccine (25 μl/mouse; CSL, Parkville, Victoria, Australia) on days 7 and 21. On day 28, mice were killed humanely to collect peritoneal macrophages, blood, spleens and intestinal washings. Food intake and body weight were measured at weekly intervals.

All protocols for animal use were approved by the Massey University Animal Care and Ethics Committee.

### *Blood*

Mice were anaesthetized with the inhalation anaesthetic, Forane (Abbott Laboratories, Abbott Park, IL, USA). Blood was collected by cardiac puncture using a hypodermic needle.

### *Peritoneal macrophages*

Resident peritoneal cells were collected by washing the peritoneal cavity of each mouse with 5 ml RPMI-1640 (Sigma). The cells were washed twice in RPMI-1640 medium containing fetal calf serum (100 ml/l), 100 IU/ml penicillin and 100 μg/ml streptomycin (complete RPMI-1640 medium) and resuspended in fresh medium at $10^6$ cells/ml.

### *Spleen-cell suspensions*

Spleens were removed aseptically from mice and placed individually into 2 ml complete RPMI-1640 medium. Single-cell suspensions were prepared by chopping the spleens into small pieces with sterile scissors and then forcing the spleen tissue up and down through a 1 ml syringe. The resulting suspension was then transferred to a tube containing 5 ml complete RPMI-1640 and centrifuged at 169 ***g*** for 10 min. The cell pellet was resuspended in ACK lysis buffer (Tris-$NH_4Cl$) and incubated for 5 min with occasional mixing to lyse the erythrocytes. After washing twice in complete RPMI-1640, the cell viability was determined by flow cytometry (after staining with propidium iodide) and was found to be greater than 95 % in all cases. The cells were adjusted to a final concentration of $2 \times 10^6$ cells/ml in complete RPMI-1640.

### *Proliferation assay*

*In vitro* proliferation responses of spleen cells to mitogens were determined using a commercial cell proliferation kit (Boehringer Mannheim, Mannheim, Germany) as previously described (Cross & Gill, 1999). Briefly, $10^5$ cells in 50 μl complete RPMI-1640 medium were added in

triplicate to the wells of a ninety-six-well flat-bottomed tissue culture plate (Nunc, Roskilde, Denmark) and cultured in the presence or absence of T- and B-cell mitogens. Mitogens (50 μl/well), concanavalin A (ConA, 2·5 μg/ml; Sigma) and lipopolysaccharide (5 μg/ml; Sigma) were added to wells at a predetermined optimal concentration. Control wells received 50 μl complete RPMI-1640 medium. The cells were cultured for 72 h at 37° in a humidified $CO_2$–air (5 : 95, v/v) atmosphere, and cell proliferation over the final 18 h of culture was determined by measuring the incorporation of 5-bromo-2′-deoxyuridine using peroxidase-conjugated anti-bromodeoxyuridine antibodies and a peroxidase substrate system. The absorbance of each well was read at 450 nm using a CERES 900C Bio-Tek microtitre plate reader (Biotek Instruments Inc., Winooski, VT, USA) and the results expressed as mean absorbance values with their standard errors for triplicate samples.

### *Estimation of antibody responses*

An ELISA was used to determine antibody responses to cholera toxin (CT) and tetanus toxoid (TT). The wells of a ninety-six-well microtitre plate were coated with CT (10 μg/ml; Sigma) or tetanus antigen (1μg/ml; CSL) in 0·05 M-carbonate–bicarbonate buffer (pH 9·6) by incubating overnight at 4°. The wells were washed three times with PBS containing 0·5 ml Tween-20/l. Serum or intestinal fluid samples diluted in PBS were added to triplicate wells (100 μl/well) and the plates incubated overnight at 4°. Serial dilutions of hyperimmune serum, obtained from mice repeatedly immunized with CT or TT, were included onto each plate as standards. The plates were washed with PBS with Tween-20 and 100 μl alkaline phosphatase-conjugated rat anti-mouse immunoglobulin (Silenus, Boronia, Victoria, Australia) diluted in sample buffer added to each well. After incubation for 1 h at 37° and washing three times with PBS with Tween-20, 100 μl/well of alkaline phosphate substrate (BioRad, Regents Park, NSW, Australia) was added. The absorbance was read at 405 nm using a CERES 900C BioTek microtitre plate reader.

### *Cytokines*

Spleen cell suspensions (2 ml; $4\times10^6$ cells/ml) were added to each well of a twenty-four-well plate (Costar, Cambridge, MA, USA) and cultured in the presence of ConA (1μg/ml; Sigma) for 48 h at 37°. Cell-free supernatant fractions were harvested and stored at −20° until assayed.

The presence of interleukin (IL)-4 and interferon-γ (IFN-γ) in culture supernatant fractions was determined using a sandwich ELISA based on the Pharminogen (San Diego, CA, USA) cytokine protocol. Rat anti-mouse IL-4 (Serotec, Raleigh, NC, USA) and rat anti-mouse IFN-γ (Pharminogen) monoclonal antibodies were used as the capture antibodies and biotinylated rat anti-mouse IL-4 (Pharminogen) and rat anti-mouse IFN-γ (Pharminogen) monoclonal antibody were used as the detection antibodies. Recombinant cytokine standards for IL-4 (Serotec) and IFN-γ (Life Technologies, Auckland, NZ) were included in all ELISA determinations.

### *Analysis of leucocyte subsets*

Flow cytometric analysis was used for monitoring the expression of $CD4^+$, $CD8^+$ and $CD40^+$ antigens on peripheral blood leucocytes (PBL). Staining of PBL was performed using the method of Lloyd *et al.* (1995) with some modifications. Briefly, 100 μl whole blood was incubated with 5 μl fluorescein-conjugated monoclonal antibodies to CD4 and CD8 (Serotec) or phycoerythrin-conjugated monoclonal antibodies to CD40 (Serotec). Samples were incubated on ice for 20 min and then washed twice with PBS. PBL were then fixed with 100 μl paraformaldehyde (80 ml/l) for 1 min and the erythrocytes lysed with 1 ml sterile water. After washing twice, cells were resuspended in PBS and stored in the dark (maximum 2 h) until analysed. Analysis was carried out on a FACSCalibur flow cytometer (Becton Dickinson Instruments, Cambridge, MA, USA) using an excitation wavelength of 488 nm and an emission wavelength of 525 nm for CD4 and CD8 or 575 nm for CD40.

### *Phagocytosis*

Assessment of the phagocytic capacity of PBL and peritoneal macrophages by flow cytometry was based on the method of Wan *et al*. (1993) with some modifications. Briefly for PBL, 10 μl fluorescein isothiocyanate-labelled *Escherichia coli* and 100 μl whole blood were mixed and incubated at 37° for 30 min. The PBL were fixed with 100 μl paraformaldehyde (80 ml/l) and 1 ml ice-cold water was added to lyse the erythrocytes. Samples were then centrifuged at 2700 ***g*** for 10 min and the pellet resuspended in 0·5 ml of PBS and 50 μl Trypan blue (4 g/l in PBS). The level of phagocytic activity was determined using a FACSCalibur flow cytometer (Becton Dickinson Instruments). For peritoneal macrophages, the method was as described earlier except that 100 μl peritoneal macrophages ($10^6$ cells/ml) was used, the incubation period was 20 min only and immediately following incubation 0·5 ml ice-cold PBS and 50 μl Trypan blue (4 ml/l) were added.

### *Natural killer-cell activity*

The flow cytometry method of Johann *et al*. (1995) was used to assess natural killer (NK)-cell activity of spleen cells. Cells from the mouse Moloney leukaemia cell line, YAC-1, were used as the target cells. YAC-1 cells ($0·5\times10^6$ cells/ml) were labelled with 2·5 μg/ml dioctadecyloxacarbocyanine perchlorate (D275; Molecular Probes, Eugene, OR, USA) by incubating overnight at 37°. After washing three times in complete RPMI-1640, YAC-1 cells were resuspended at a concentration of $1\times10^6$ cells/ml. Spleen lymphocyte effector cells were added in triplicate ($10^6$ cells/well) to a ninety-six-well plate (Nunc) together with target cells at $2·5\times10^4$ cells/well in a total volume of 100 μl/well (effector : target ratio 40 : 1). Samples were centrifuged (1 min, 240 ***g***) and incubated for 3 h (37°, in humidified $CO_2$–air atmosphere). At 15 min before the end of the incubation, 1 μl propidium iodide (5 mg/ml in PBS; Sigma) was added to each well to label dead cells. The level of target cell lysis was determined using a FACSCalibur flow cytometer (Becton Dickinson Instruments), and NK-cell activity was expressed as the percentage of effector cell-specific lysis.

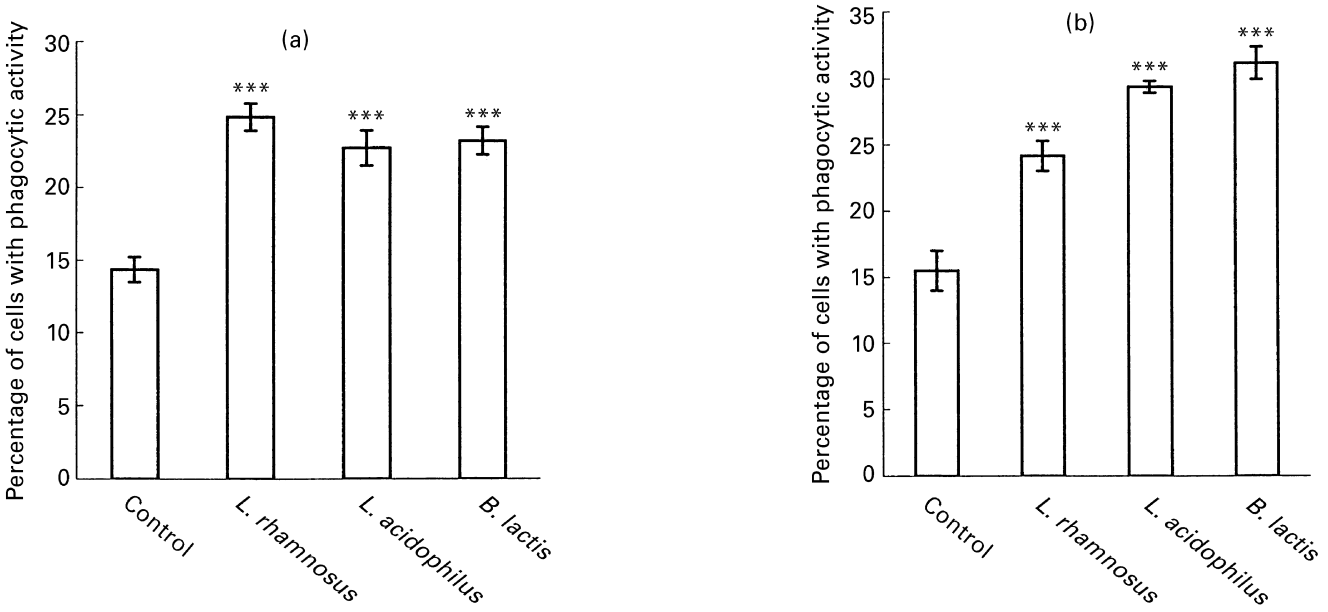


**Fig. 1.** Percentage of cells showing phagocytic activity in the peripheral blood of mice orally administered with *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) ($10^9$ colony-forming units/d) in 50 μl skimmed milk for (a) 10 or (b) 28 d. Control mice received 50 μl skimmed milk only. Peripheral blood leucocytes were incubated with FITC-labelled *Escherichia coli* and the percentage of cells with phagocytic activity determined using flow cytometry. Values are means for eighteen to twenty mice per group, with their standard errors represented by vertical bars. Mean values were significantly different from those for the control group: ***$P < 0{\cdot}001$ (ANOVA).

### *Statistical analysis*

Significant differences between the experimental and control groups were determined on log-transformed data using ANOVA (SAS ver. 6.12, Statistical Analysis Systems Institute Inc., Cary, NC, USA). Values of $P < 0{\cdot}05$ were considered significant.

## Results

### *Body weight and feed intake*

Supplementation of the diets of mice with *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) for 28 d had no significant effect on feed intake or weight gain (results not shown).

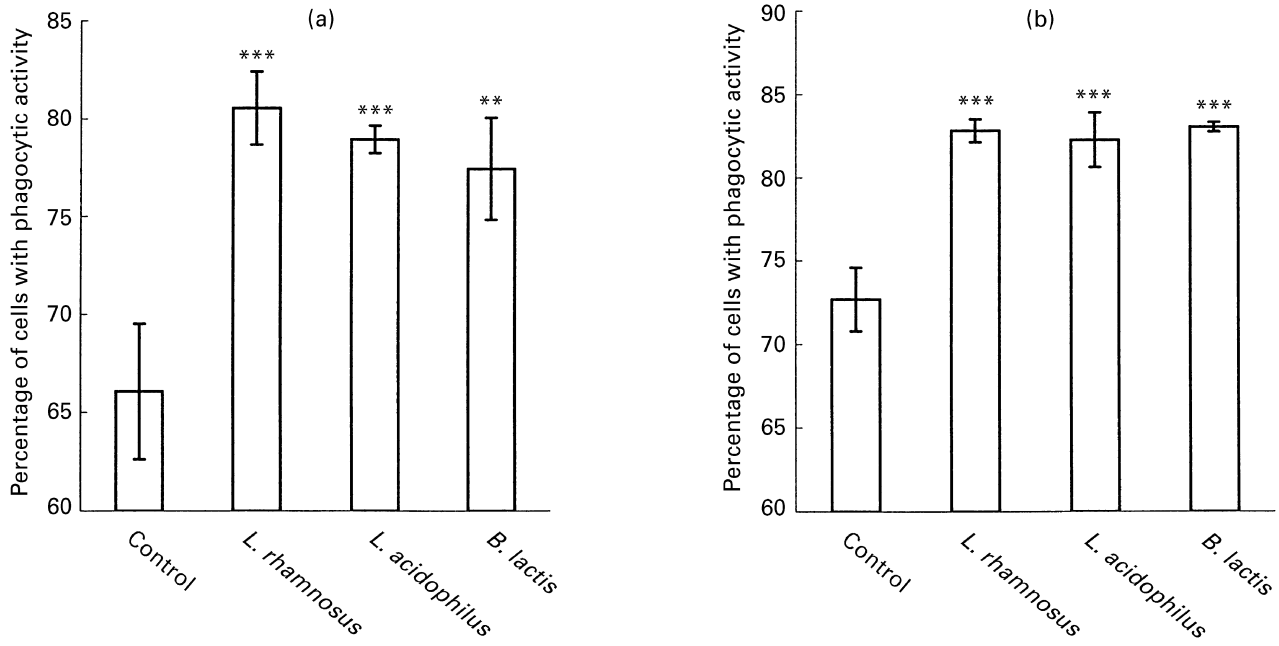


**Fig. 2.** Percentage of peritoneal macrophages showing phagocytic activity in mice orally administered with *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) ($10^9$ colony-forming units/d) in 50 μl skimmed milk for (a) 10 or (b) 28 d. Control mice received 50 μl skimmed milk only. Peritoneal macrophages were incubated with FITC-labelled *Escherichia coli* and the percentage of cells with phagocytic activity determined using flow cytometry. Values are means for eighteen to twenty mice, with their standard errors represented by vertical bars. Mean values were significantly different from those for the control group: **$P < 0{\cdot}01$, ***$P < 0{\cdot}005$.

### *Phagocytic function of peritoneal macrophages and blood leucocytes*

The phagocytic activities of PBL and peritoneal macrophages are shown in Figs. 1 and 2. Blood leucocytes and peritoneal macrophages from mice fed with *L. rhamnosus*, *L. acidophilus* or *B. lactis* for 10 or 28 d exhibited significantly greater phagocytic activity than cells from control mice. The increase in macrophage (peritoneal) phagocytic activity observed after 10 d of feeding with *L. rhamnosus*, *L. acidophilus* or *B. lactis* was not significantly different from that observed after 28 d of feeding. However, the blood leucocytes from mice fed with *L. acidophilus* or *B. lactis* for 28 d exhibited significantly higher phagocytic capacity than mice fed with the same bacteria for 10 d ($P < 0{\cdot}05$). There were no significant differences in the magnitude of phagocytosis for peritoneal macrophages between mice fed with different strains of LAB; however, levels of phagocytosis for blood leucocytes were significantly higher among mice fed with *L. acidophilus* or *B. lactis* for 28 d than among mice that received *L. rhamnosus*.

### *Antibody responses*

The effects of feeding LAB on specific antibody responses to CT (Expt 1) and TT (Expt 2) are shown in Table 1 and Fig. 3 respectively. Mice fed with *L. rhamnosus*, *L. acidophilus* or *B. lactis* for 10 d produced significantly higher levels of serum antibodies to CT compared with the control mice. The mucosal antibody responses to CT in mice fed with LAB tended to be higher, but were not significantly different from those in the control mice.

Serum antibody responses to TT were also significantly greater in mice fed with *L. rhamnosus*, *L. acidophilus* or *B. lactis* for 28 d compared with the responses in control mice.

### *Proliferation responses*

To evaluate the effect of LAB feeding on T- and B-cell function in mice, proliferation responses of spleen cells to the T-cell mitogen, ConA and the B-cell mitogen, lipopolysaccharide were measured *in vitro*.

As shown in Table 2, the proliferative responses of spleen cells from mice fed with *B. lactis* for 10 or 28 d to stimulation with ConA, were significantly higher than those from the control mice. ConA-induced proliferative

**Table 1.** Effect of feeding *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) for 10 d on serum and mucosal antibody responses to cholera toxin in mice†

(Mean values with their standard errors for twenty mice per group)

| | Serum antibody concentration (units/ml) | | Mucosal secrections antibody concentration (units/ml) | |
|---|---|---|---|---|
| Treatment | Mean | SEM | Mean | SEM |
| Control | 80·3 | 7·2 | 1346·9 | 212·3 |
| *L. rhamnosus* (HN001) | 118·5* | 12·3 | 1573·6 | 231·8 |
| *L. acidophilus* (HN017) | 134·6** | 29·3 | 1540·5 | 268·2 |
| *B. lactis* (HN019) | 201·1** | 56·1 | 1508·8 | 208·0 |

Mean values were significantly different from those for the control group: *$P <$ 0·05, **$P <$ 0·01 (ANOVA).

† For details of diets and procedures, see pp. 168–170.

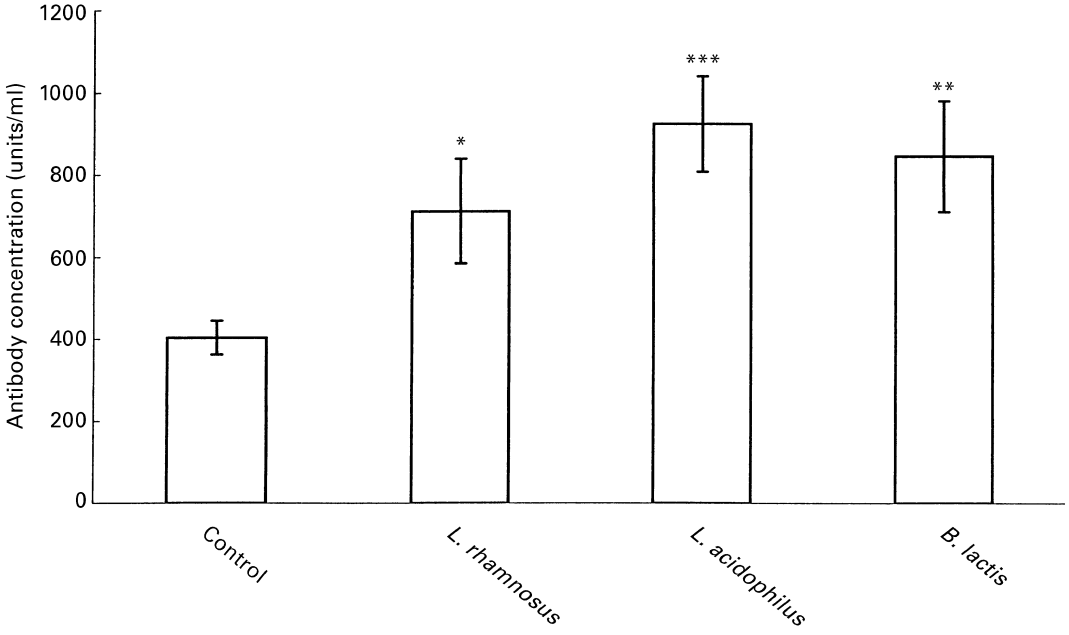


**Fig. 3.** Serum antibody response to tetanus toxoid (TT) in mice orally administered with *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) ($10^9$ colony-forming units/d) in 50μl skimmed milk for 28 d. Control mice received 50 μl skimmed milk only. Mice were immunized subcutaneously with tetanus vaccine on days 7 and 21. The concentration of anti-TT antibody in serum was determined on day 28 using an ELISA. Values are means for eighteen mice per group, with their standard errors represented by vertical bars. Mean values were significantly different from those for the control group: *$P <$ 0·05, **$P <$ 0·005, ***$P <$ 0·001.

**Table 2.** Effect of feeding *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) for 10 or 28 d on proliferative responses of spleen cells to lipopolysaccharide (LPS) and concanavalin A (ConA) in mice†

(Mean values with their standard errors for eighteen to twenty mice per group)

| | | Cell proliferation (absorbance) (10 d feeding) | | Cell proliferation (absorbance) (28 d feeding) | |
|---|---|---|---|---|---|
| Mitogen | Group | Mean | SEM | Mean | SEM |
| LPS | Control | 0·98 | 0·06 | 0·79 | 0·05 |
| | *L. rhamnosus* (HN001) | 1·37* | 0·17 | 1·02* | 0·11 |
| | *L. acidophilus* (HN017) | 1·29* | 0·11 | 0·98* | 0·04 |
| | *B. lactis* (HN019) | 1·32* | 0·09 | 0·96 | 0·06 |
| ConA | Control | 1·42 | 0·12 | 1·35 | 0·13 |
| | *L. rhamnosus* (HN001) | 1·64 | 0·10 | 1·76*** | 0·10 |
| | *L. acidophilus* (HN017) | 1·61 | 0·11 | 1·59* | 0·04 |
| | *B. lactis* (HN019) | 1·82* | 0·15 | 1·68** | 0·05 |

Mean values were significantly different from those for the control group: *$P < 0·05$, **$P < 0·01$, ***$P < 0·005$ (ANOVA).

† For details of diets and procedures, see pp. 168–170.

responses of spleen cells from mice given *L. rhamnosus* and *L. acidophilus* for 10 d were higher, but not significantly different from those of control mice. After 28 d, however, mice fed with *L. rhamnosus* and *L. acidophilus* exhibited significantly greater proliferation responses to ConA compared with the control mice.

For lipopolysaccharide, significantly higher proliferative responses were observed in mice given *L. rhamnosus*, *L. acidophilus* or *B. lactis* for both 10 and 28 d compared with the control mice (Table 2).

### *Lymphocyte subsets*

Table 3 shows the percentages of lymphocyte subsets in the peripheral blood of control mice and mice fed with LAB for different lengths of time. Ingestion of *L. rhamnosus*, *L. acidophilus* or *B. lactis* for 10 d had no noticeable effect on the populations of $CD4^+$, $CD8^+$ and $CD40^+$ cells. However, a slight decrease in the percentage of $CD4^+$ cells and an increase in the percentage of $CD40^+$ cells were observed in mice fed with different LAB for 28 d.

### *Cytokine production*

ConA-induced IFN-γ production was significantly higher in spleen cells from mice fed with *L. rhamnosus* and *L. acidophilus* for different lengths of time (Fig. 4). However, the production of IFN-γ by spleen cells from mice given *B. lactis* did not differ significantly from that of control mice. There was no significant difference in IL-4 production between control mice and mice with fed with different LAB for either 10 or 28 d.

### *Natural killer-cell activity*

As shown in Table 4, feeding with LAB had a stimulatory effect on NK-cell activity of splenocytes. Spleen cells from mice fed with different LAB exhibited higher NK-cell cytotoxic activity (10–27 %) against the target cells, YAC-1, compared with that of control mice. However, significantly higher responses ($P < 0·05$), compared with the control group, were only noted for mice fed with *L. rhamnosus*.

## Discussion

Optimally functioning innate (non-specific or natural) and acquired (specific) immune systems are essential for host defence against invading pathogens and spontaneously

**Table 3.** Effect of feeding *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) for 10 or 28 d on lymphocyte subsets in mice*

(Mean values with their standard errors for eighteen to twenty mice per group)

| | $CD4^+$ cells (%) | | | | $CD8^+$ cells (%) | | | | $CD40^+$ cells (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10 d | | 28 d | | 10 d | | 28 d | | 10 d | | 28 d | |
| Group | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| Control | 39·11 | 3·11 | 51·78 | 2·21 | 12·09 | 0·79 | 16·16 | 1·18 | 36·32 | 3·88 | 29·94 | 2·95 |
| *L. rhamnosus* (HN001) | 43·00 | 2·04 | 6·43 | 5·04 | 16·14 | 2·23 | 14·15 | 0·48 | 34·55 | 2·67 | 32·17 | 3·85 |
| *L. acidophilus* (HN017) | 40·24 | 1·93 | 43·85 | 5·12 | 14·32 | 1·27 | 15·81 | 0·36 | 37·36 | 3·61 | 35·93 | 5·82 |
| *B. lactis* (HN019) | 40·50 | 3·06 | 45·24 | 2·05 | 14·54 | 1·11 | 16·02 | 1·36 | 39·10 | 3·35 | 38·39 | 3·71 |

* For details of diets and procedures, see pp. 168–170.

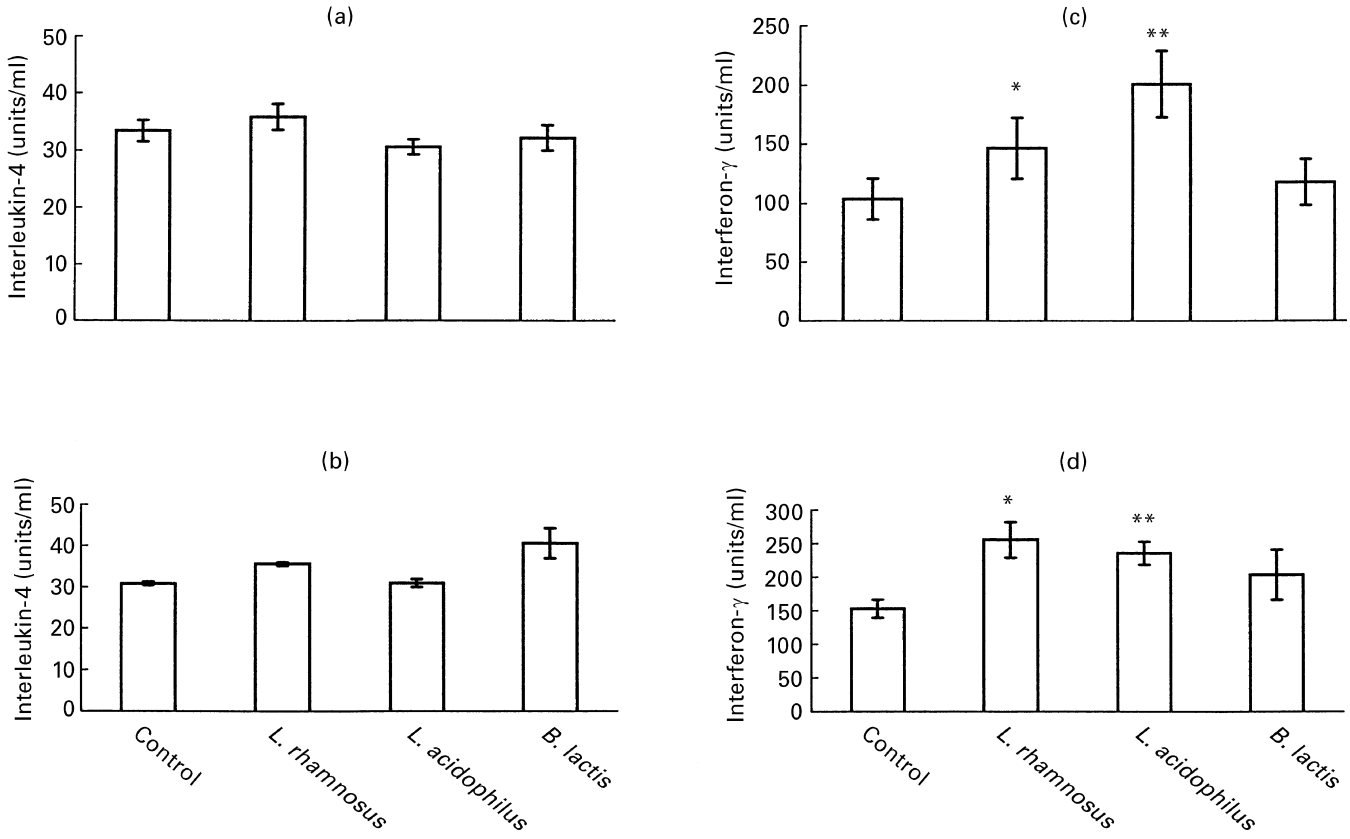


**Fig. 4.** Production of interleukin-4 (a, b) and interferon-γ (c, d) by spleen cells from mice orally administered with *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) ($10^9$ colony-forming units/d) in 50 μl skimmed milk for (a, c) 10 or (b, d) 28 d. Control mice received 50 μl skimmed milk only. Spleen cells were cultured *in vitro* with concanavalin A for 48 h. Concentration of cytokines in culture supernatant fractions were measured by sandwich ELISA and the results are expressed as units/ml. Values are means for eighteen to twenty mice per group, with their standard errors represented by vertical bars. Mean values were significantly different from those for the control group: $*P < 0{\cdot}05$, $**P < 0{\cdot}01$.

developing cancers. The results of our studies demonstrate that several indices of natural and acquired immunity were enhanced in healthy mice fed with *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019).

Phagocytic (monocytes, macrophages, polymorphonuclear leucocytes) and NK cells are the major effectors of natural immunity. Feeding of mice with *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) resulted in enhanced phagocyte function. This was shown by a significant increase (66–100 %) in the phagocytic activity of PBL (monocytes and polymorphonuclear cells) of mice fed with different LAB compared with the control mice. The phagocytic activity of peritoneal macrophages was also significantly increased (16–23 %). A greater increase in the phagocytic activity of PBL than peritoneal macrophages could be due to the presence of polymorphonuclear cells in blood; polymorphonuclear cells represent 25–35 % of the leucocytes in mouse blood (Plata & Murphy, 1972). Significantly higher phagocytic activity of granulocytes compared with monocytes in human subjects given *L. acidophilus* (La1) and *B. bifidum* (Bb12) has previously been reported (Schiffrin *et al.* 1997). In the present study, an increase in phagocyte function became significant as early as 10 d of feeding, the earliest time of examination, and was maintained at a similar level for the period of feeding. Enhanced phagocytic activity of PBL or peritoneal macrophages from human subjects (Mikes *et al.* 1995; Schiffrin *et al.* 1997) and animals (Perdigon *et al.* 1986; Paubert-Braquet *et al.* 1995) given dietary LAB has previously been demonstrated. It has also been shown that the level of enhancement depends on the strain, dose and viability of LAB used (Gill, 1998). Whether the increase in phagocytic capacity is maintained after cessation of LAB feeding is not clear. Schiffrin *et al.* (1997) noted that the

**Table 4.** Effect of feeding *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) or *Bifidobacterium lactis* (HN019) for 28 d on natural killer-cell activity in mice†

(Mean values with their standard errors for eighteen mice per group)

| | Natural killer-cell activity (% cell lysis) | |
|---|---|---|
| Treatment | Mean | SEM |
| Control | 8·86 | 0·61 |
| *L. rhamnosus* (HN001) | 11·53* | 1·14 |
| *L. acidophilus* (HN017) | 9·86 | 0·82 |
| *B. lactis* (HN019) | 10·49 | 0·78 |

Mean value was significantly different from that for the control group: $*P<0{\cdot}05$ (ANOVA).

† For details of diets and procedures, see pp. 168–170.

increased phagocytic activity in human subjects persisted for 6 weeks after ingestion of *L. acidophilus* (La1) and *B. bifidum* (Bb12).

NK-cell activity was also enhanced in mice fed with different LAB although significant differences occurred only for *L. rhamnosus* (HN001). This indicates strain-dependent variation in the ability of LAB to influence NK-cell function. Whether the increase in NK-cell activity reflected an increase in the percentage of NK cells (De Simone *et al.* 1991) or functional enhancement at a cellular level is not known. Increased NK-cell activity in mice injected with *L. casei* (Kato *et al.* 1981) and of PBL co-cultured with ConA and yoghurt containing live LAB have also been reported (De Simone *et al.* 1986). To our knowledge, this is the first report on the effect of orally administered LAB on NK-cell function.

Lymphocyte proliferation responses to mitogens are widely used to assess T- and B-cell function (Gill *et al.* 1992). In the present study, mice fed with LAB also exhibited enhanced T- and B-cell functions as indicated by elevated proliferation responses to the T-cell mitogen, ConA, and the B-cell mitogen, lipopolysaccharide. All strains were able to enhance these functions equally. As the proportion of T cells was not significantly different in mice fed with different LAB than in control mice, it could be concluded that an increased proliferation response to ConA was due to an augmented T-cell function. Whether the increase in B-cell function was due to a relative increase in the proportion of B cells or enhancement of cell function is not clear.

T cells are the main effectors and regulators of cell-mediated immunity (Roitt *et al.* 1985). On activation by antigen or pathogen, T cells synthesize and secrete a variety of cytokines that serve as growth, differentiation and activation factors for other immunocompetent cells. Recent studies have shown that T cells can be subdivided into two functional types, Th1 and Th2, based on their cytokine profile (Mossman & Coffman, 1989). Th1 cells produce IL-2, IFN-$\gamma$ and tumour necrosis factor and are vital for cell-mediated immunity. On the other hand, Th2 cells predominantly produce IL-4, IL-5 and IL-10 and are associated with humoral immunity and allergic responses. In the present study, spleen cells from mice fed with *L. rhamnosus* and *L. acidophilus* produced significantly greater amounts of IFN-$\gamma$ following stimulation with ConA. IL-4 production was not influenced by LAB. This clearly shows that feeding with *L. rhamnosus* and *L. acidophilus* results in the selective activation of Th1 cells. Increased production of IFN-$\gamma$ in human subjects following ingestion of LAB or yoghurt has been reported by De Simone *et al.* (1991), Solis Pereyra & Lemonnier (1991) and Halpern *et al.* (1991). To date, however, no studies have reported the effect of LAB consumption on Th2 cytokines. Furthermore, it is interesting to note that the ability to stimulate IFN-$\gamma$ production was species dependent. *L. rhamnosus* and *L. acidophilus* were more efficient at stimulating IFN-$\gamma$ production than *B. lactis*. The reasons for this variation are not known.

Although many cell types are known to produce IFN-$\gamma$ following appropriate stimulation, T cells appear to be the major source. Since the proportion of T cells was the same in mice fed *L. rhamnosus* or *L. acidophilus* and in control mice, it could be concluded that an increase in IFN-$\gamma$ production by spleen cells in LAB-fed mice may, therefore, reflect a selective influence of LAB on Th1 cell activation. Higher lymphocyte proliferation responses and increased production of IFN-$\gamma$ are consistent with this conclusion.

The physiological significance of enhanced IFN-$\gamma$ production to human health is not known. Studies in experimental animals have shown that IFN-$\gamma$ can inhibit viral replication, enhance antigen-presentation function of macrophages, modify the expression of major histocompatability complex antigens, enhance T-cell function, activate macrophages and improve the effectiveness of vaccines (Murray, 1988). Increased IFN-$\gamma$ production in the present study was accompanied by enhanced T-cell responses to ConA, increased phagocytic activity and superior antibody responses to immunizations with CT and TT.

It is also well documented that IL-4 regulates the production of immunoglobulin E (IgE) and an elevated level of IgE is an important indicator of allergy (Mossman & Coffman, 1989; Mekori, 1996; Ryan, 1997). In contrast, IFN-$\gamma$ inhibits IL-4 secretion and suppresses IgE production (Thyphronitis *et al*. 1989). The results of the present study therefore suggest that *L. rhamnosus, L. acidophilus* and *B. lactis* may inhibit IgE-mediated allergic responses through selective stimulation of Th1 cells. Suppression of antigen-induced IgE production by murine splenocytes *in vitro* in the presence of heat-killed *L. casei* (strain Shirota) has been recently reported by Shida *et al.* (1998).

Furthermore, supplementation of the diet with *L. rhamnosus, L. acidophilus* and *B. lactis* was found to enhance antibody responses to orally as well as systemically administered immunogens. The antibody responses of LAB-fed mice to CT and TT were significantly higher (45–129 %) than those of control mice. The mucosal antibody responses to CT of mice fed with LAB were also higher than those of controls but did not reach significant levels. Whether this was due to the immunization regimen used (two immunizations over 7 d) or that feeding with LAB was too short (10 d) to realise the full immunopotentiating effect of LAB is not clear. Gill (1998) reported significantly higher mucosal antibody responses to orally administered CT in mice fed on diets containing *L. rhamnosus* (HN001) for 14 d. Induction of both systemic and mucosal antibody responses following oral immunization with CT is consistent with the observations of Xu-Amano *et al.* (1994) and reflects the extraintestinal migration of antigen-sensitive lymphocytes from the gut-associated lymphoid tissue to various mucosal and systemic effector sites (Phillips-Quagliata & Lamm, 1994). Augmentation of specific serum and/or mucosal antibody responses to antigens, vaccines, and bacterial and viral pathogens have been observed in animals and human subjects given LAB (Nader de Macias *et al.* 1992; Perdigon & Alvarez, 1992; Link Amster *et al*. 1994; Isolauri *et al.* 1995; Majamaa *et al.* 1995). It has been shown that different strains of LAB vary greatly in their ability to enhance humoral immunity (Perdigon & Alvarez, 1992). In the present study, however, *L. rhamnosus* (HN001), *L. acidophilus* (HN017) and *B. lactis* (HN019) were equally effective in enhancing specific antibody responses.

Enhanced phagocyte function, NK-cell activity, T- and B-cell function, and antigen-specific antibody responses are likely to result in an increased resistance to invading pathogens and malignancy. In a preliminary study, mice fed on diets containing *L. rhamnosus* or *B. lactis* were found to exhibit increased resistance to oral infection with *Salmonella typhimurium.* Furthermore, the level of resistance to *S. typhimurium* was found to be directly correlated with phagocytic capacity of PBL and peritoneal macrophages, and spleen-cell proliferative responses to a T-cell mitogen, ConA (HS Gill, Q Shu and KJ Rutherfurd, unpublished results). The ability of some strains of LAB to confer protection against bacterial and viral infections and against artificially induced cancers has been reported by several investigators (Gill, 1998).

The precise mechanisms by which LAB stimulate the immune system are not fully understood. It is possible that when present in large numbers, LAB or their products are able to gain access to the gut-associated lymphoid tissue, especially Peyer's patches and/or to the systemic immune system. The ability of Peyer's patches cells to produce specific antibodies to LAB (Yasui *et al.* 1989) and to exhibit increased proliferative responses to T- and B-cell mitogens (De Simone *et al.* 1987), and the translocation of LAB to mesenteric lymph nodes and Peyer's patches of mice orally administered with LAB (Claassen *et al.* 1995) support this possibility. Furthermore, the intestinal epithelium contains a variety of immunoregulatory cells (Lefrancois, 1994) and thus the possibility that LAB and their products may exert their influence through these cells also exists. The interaction between LAB or LAB-derived products and immunocompetent cells (e.g. macrophages, T cells), results in the secretion of cytokines (Gill, 1998) that are known to have a multitude of effects on the functioning of the immune system. Specific receptors for peptidoglycans, cell-wall components of LAB on lymphocytes and macrophages, has also been reported (Dziarski, 1991).

In summary, the results of this study indicate that ingestion of *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) is able to enhance several indices of natural and acquired immunity (phagocytic activity of PBL and macrophages, NK-cell activity, IFN-$\gamma$ production, antibody responses to orally and systemically administered antigens) in healthy mice. Thus, supplementation with *L. rhamnosus* (HN001), *L. acidophilus* (HN017) or *B. lactis* (HN019) may be beneficial for optimizing and/or enhancing immunocompetence in healthy, immunosuppressed or immunocompromised subjects. This is supported by the results of our recent clinical studies (Arunachalam *et al.* 1999; HS Gill, KJ Rutherfurd and PK Gopal, unpublished results) which showed that dietary consumption of these bacteria can enhance a range of immune functions in healthy subjects.

## Acknowledgements

We thank Sarah Blackburn, Daniel Johnson, Kim Kennedy and Anne Broomfield for skilled technical assistance, Quan Shu for statistical analysis and John Smart for helpful discussions.

## References

Arunachalam K, Gill HS & Chandra RK (1999) Enhancement of natural immune function by dietary consumption of *Bifidobacterium lactis* (HN019). *European Journal of Clinical Nutrition* (In the Press).

Baradana EJ (1985) Recent developments in immunomodulatory therapy. *Journal of Allergy and Clinical Immunology* **5**, 423–437.

Claassen E, Van Winsen R, Posno M & Boersma WJA (1995) New and safe oral live vaccines based on *Lactobacillus*. In *Advances in Mucosal Immunology*, pp. 1553–1558 [PL Ogra, J Mestecky, ME Lamm, W Strober, JR McGhee and J Bienenstock, editors]. New York, NY: Plenum Press.

Cross ML & Gill HS (1999) Modulation of immune function by a modified bovine whey protein concentrate. *Immunology and Cell Biology* **77**, 288–296.

De Simone C, Bianchi Salvadori B, Negri R, Ferrazzi M, Baldinelli L & Vesely R (1986) The adjuvant effect of yogurt on production of gamma-interferon by ConA-stimulated human peripheral blood lymphocytes. *Nutrition Reports International* **33**, 419–433.

De Simone C, Rosati E, Moretti S, Bianchi Salvadori B, Vesely R & Jirillo E (1991) Probiotics and stimulation of the immune response. *European Journal of Clinical Nutrition* **45**, Suppl., 32–34.

De Simone C, Vesely R, Negri R, Bianchi Salvadori B, Zanzoglu S, Cilli A & Lucci L (1987) Enhancement of immune response of murine Peyer's patches by a diet supplemented with yoghurt. *Immunopharmacology and Immunotoxicology* **9**, 87–100.

Dziarski R (1991) Demonstration of peptidoglycan-binding sites on lymphocytes and macrophages by photoaffinity cross-linking. *Journal of Biological Chemistry* **266**, 4713–4718.

Edelman AS & Zolla-Pazner S (1989) AIDS: a syndrome of immune dysregulation, dysfunction and deficiency. *FASEB Journal* **3**, 79–84.

Gill HS (1998) Stimulation of the immune system by lactic cultures. *International Dairy Journal* **8**, 535–544.

Gill HS, Watson DL & Brandon MR (1992) *In vivo* inhibition by a monoclonal antibody to $CD4^+$ T cells of humoral and cellular immunity in sheep. *Immunology* **77**, 38–42.

Goldin BR (1998) Health benefits of probiotics. *British Journal of Nutrition* **80**, Suppl. 2, S203–S207.

Goodwin JS (1995) Decreased immunity and increased morbidity in the elderly. *Nutrition Reviews* **53**, S41–S46.

Halpern GM, Vruwink KG, Van De Water J, Keen CL & Gershwin ME (1991) Influence of long-term yoghurt consumption in young adults. *International Journal of Immunotherapy* **7**, 205–210.

Isolauri E, Joensus J, Suomalainen H, Luomala M & Vesikari T (1995) Improved immunogenicity of oral DxRRX reabsorbant rotavirus vaccine by *Lactobacillus casei* GG. *Vaccine* **13**, 310–312.

Johann S, Blümel G, Lipp M & Förster R (1995) A versatile flow cytometry-based assay for the determination of short- and long-term natural killer cell activity. *Journal of Immunological Methods* **185**, 209–216.

Kato I, Yokokawa T & Mutai M (1981) Antitumor activity of *Lactobacillus casei* in mice. *Japanese Journal of Cancer Research (Gann)* **72**, 517–523.

Khansari DN, Murgo AJ & Faith AE (1990) Effects of stress on the immune system. *Immunology Today* **11**, 170–175.

Lefrancois L (1994) Basic aspects of intraepithelial lymphocyte immunobiology. In *Handbook of Mucosal Immunology*, pp. 287–290 [PL Ogra, J Mestecky, ME Lamm, W Strober, JR McGhee and J Bienenstock, editors]. San Diego, CA: Academic Press.

Link-Amster H, Rochat F, Saudan KY, Mignot O & Aeschlimann JM (1994) Modulation of a specific humoral immune response and changes in intestinal flora mediated through fermented milk intake. *FEMS Immunology and Medical Microbiology* **10**, 55–64.

Lloyd JB, Gill HS & Husband AJ (1995) The effect of storage on immunophenotyping of sheep lymphocyte by flow cytometry. *Veterinary Immunology and Immunopathology* **47**, 131–142.

Majamaa H, Isolauri E, Saxelin M & Vesikari T (1995) Lactic acid bacteria in the treatment of acute rotavirus gastroenteritis. *Journal of Pediatric Gastroenterology and Nutrition* **20**, 333–338.

Mekori YA (1996) Introduction to allergic diseases. *Critical Reviews in Food Science and Nutrition* **36**, S1–S18.

Mikes Z, Ferenicik M, Jahnova E, Ebringer L & Ciznar I (1995) Hypocholesterolemic and immunostimulatory effects of orally applied *Enterococcus faecium* M-74 in man. *Folia Microbiologica* **40**, 639–646.

Mossmann TR & Coffman RL (1989) Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Review of Immunology* **7**, 145–173.

Murray HW (1988) Interferon-gamma, the activated macrophages, and host defense against microbial challenge. *Annals of International Medicine* **108**, 595–608.

Nader de Macias ME, Apella MC, Romero NC, Gonzalez SN & Oliver G (1992) Inhibition of *Shigella sonnei* by *Lactobacillus casei* and *Lactobacillus acidophilus*. *Journal of Applied Bacteriology* **73**, 407–411.

Nussler AK & Thomson AW (1992) Immunomodulatory agents in the laboratory and clinic. *Parasitology* **105**, S5–S23.

Paubert-Braquet M, Gan X-H, Gaudichon C, Hedef N, Serikoff A, Bouley C, Bonavida B & Braquet P (1995) Enhancement of host resistance against *Salmonella typhimurium* in mice fed a diet supplemented with yogurt or milks fermented with various *Lactobacillus casei* strains. *International Journal of Immunotherapy* **11**, 153–161.

Pawelec G, Adibzadeh M, Pohla H & Schaudt K (1995) Immunosenescence: aging of the immune system. *Immunology Today* **16**, 420–422.

Perdigon G & Alvarez S (1992) Probiotics and the immune state. In *Probiotics*, pp. 146–176 [R Fuller, editor]. London: Chapman & Hall.

Perdigon G, de Marcias MEN, Alvarez S, Oliver G & de Ruiz Holgado AP (1986) Effect of perorally administered lactobacilli on macrophage activation in mice. *Infection and Immunity* **53**, 404–410.

Phillips-Quagiliata JM & Lamm ME (1994) Lymphocyte homing to mucosal effector sites. In *Handbook of Mucosal Immunology*, pp. 287–290 [PL Ogra, J Mestecky, ME Lamm, W Strober, JR McGhee and J Bienenstock, editors]. San Diego, CA: Academic Press.

Plata EJ & Murphy WH (1972) Growth and hematologic properties of the BALB/wm strain of inbred mice. *Laboratory Animal Science* **22**, 712–720.

Prasad J, Gill HS, Smart JB & Gopal PK (1999) Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics. *International Dairy Journal* **8**, 993–1002.

Roitt I, Brostoff J & Male D (1989) *Immunology*, 2nd ed. London: Gower Medical Publishing.

Ryan JJ (1997) Interleukin-4 and its receptors: essential mediators of the allergic response. *Journal of Allergy and Clinical Immunology* **99**, 1–6.

Salminen S, Ouwehand AC & Isolauri E (1998) Clinical applications of Probiotic bacteria. *International Dairy Journal* **8**, 563–572.

Schiffrin EJ, Brassart D, Servin A, Rochat F & Donnet-Hughes A (1997) Immune modulation of blood leukocytes in humans by lactic acid bacteria: criteria for strain selection. *American Journal of Clinical Nutrition* **66**, 515S–520S.

Shida K, Makino K, Morishita A, Takamizawa K, Hachimura S, Ametani A, Sato T, Kumagai Y, Habu S & Kaminogawa S (1998) *Lactobacillus casei* inhibits antigen-induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. *International Archives of Allergy and Immunology* **115**, 278–287.

Solis Pereyra B & Lemonnier D (1991) Induction of 2-5A synthetase activity and interferon in humans by bacteria used in dairy products. *European Cytokine Network* **2**, 137–140.

Thyphronitis G, Tsokos GC, June CH, Levine AD & Finkelmen FD (1989) IgE secretion by Epstein-Bar virus-infected purified human B lymphocytes is stimulated by interleukin 4 and suppressed by interferon $\gamma$. *Proceedings of the National Academy of Sciences USA* **86**, 5580–5584.

Wan CP, Park CS & Lau BHS (1993) A rapid and simple microfluorometric phagocytosis assay. *Journal of Immunological Methods* **162**, 1–7.

Woodward B (1998) Protein, calories and immune defenses. *Nutrition Reviews* **56**, S84–S92.

Xu-Amano J, Jackson RJ, Fujihashi K, Kiyono H, Staats HF & McGhee JR (1994) Helper Th1 and Th2 cell responses following mucosal or systemic immunization with cholera toxin. *Vaccine* **12**, 903–911.

Yasui H, Mike A & Ohwaki M (1989) Immunogenicity of *Bifidobacterium breve* and change in antibody production in Peyer's patches after oral administration. *Journal of Dairy Science* **72**, 30–35.

Zinkernagal RM & Hengartner H (1997) Antiviral immunity. *Immunology Today* **18**, 258–259.