



The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans

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Objective: To determine the effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota (*L. casei* Shirota) on the composition and metabolic activities of the intestinal microflora, and immune parameters in humans.

Subjects: Twenty healthy male subjects aged 40–65 years were selected.

Design: A placebo-controlled trial was performed in which 10 subjects were randomly assigned to a control and 10 to a treatment group. During the first and last two weeks of the 8-week study the subjects received a strictly controlled diet without fermented products. The same controlled diet was given during the intermediate 4-week test period but then the treatment group received three times daily 100 ml of fermented milk containing 10⁹ CFU *L. casei* Shirota/ml, whereas the same amount of unfermented milk was given to the subjects in the control group.

Results: In comparison to the control group, the consumption of *L. casei* Shirota-fermented milk resulted in an increase of the *Lactobacillus* count in the faeces in which the administered *L. casei* Shirota was predominant at the level of 10⁷ CFU/g wet faeces. This was associated with a significant increase in *Bifidobacterium* counts ($P < 0.05$). Some shifts in the other bacterial species were found, such as a decreased number of *Clostridium*; however the differences were not statistically different between the treatment and the control groups.

The β -glucuronidase and β -glucosidase activities per 10¹⁰ bacteria decreased significantly ($P < 0.05$) at the second week of the 4-week test period with the consumption of *L. casei* Shirota-fermented milk. Furthermore, the consumption of the fermented milk product resulted in a slight but significant increase in the moisture content of the faecal samples ($P < 0.05$). No treatment effects were observed for any of the immune parameters measured (including natural killer (NK) cell activity, phagocytosis and cytokine production).

Conclusions: The results suggest that consumption of *L. casei* Shirota-fermented milk is able to modulate the composition and metabolic activity of the intestinal flora and indicate that *L. casei* Shirota-fermented milk does not influence the immune system of healthy immunocompetent males.

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Descriptors: fermented milk; immune system; intestinal microflora; lactic acid bacteria; *Lactobacillus casei*

Introduction

There is growing interest in the specific health effects of fermented milk products containing specific viable probiotic lactic acid bacteria. It has appeared that many intestinal disturbances may, among other causes, be related to altered gut mucosal barrier functions and that probiotics offer new dietary alternatives for the stabilisation of the intestinal microflora (reviewed by Havenaar & Huis in 't Veld, 1992; Marteau *et al*, 1993; Sanders, 1995; Salminen *et al*, 1996).

Consumption of lactobacilli can lead to an increased host resistance against pathogens. This may be due to improved competition between beneficial bacteria, selectively stimulated by the probiotic, and pathogenic bacteria or to immunomodulation. The immunomodulating properties of lactobacilli and the possible mechanisms and effects in relation to intestinal infections have been reviewed by Havenaar & Spanhaak (1994). In mouse experiments (Perdigon *et al*, 1990; Pouwels *et al*, 1996) as well as in human studies (DeSimone *et al*, 1988; Isolauri *et al*, 1991; Kaila

et al, 1992), the oral intake of lactobacilli resulted in stimulation of macrophages, lymphocytes and natural killer (NK) cells, higher production of γ -interferon and significantly higher secretory IgA responses against pathogenic agents (*Salmonella*, Rotavirus).

Experiments in mice have shown that the growth as well as the metastasis of tumours can be inhibited by a *Lactobacillus casei* strain (Matsuzaki *et al*, 1985; Asano *et al*, 1986; Kato *et al*, 1994). However, the effects are dependent on the strain of lactobacillus, the method of administration, and the type of tumour cells. Epidemiological research indicates that the consumption of fermented milk products is related to a decreased relative risk of breast cancer in women (Lé *et al*, 1986; Van 't Veer *et al*, 1989). Although the underlying mechanisms are not known, it is suggested that inactivation or inhibition of the formation of carcinogens in the intestinal tract is induced (Fernandes *et al*, 1987). Furthermore, enhancement or stimulation of immune functions have been described, which may also contribute to a decrease in the risk of the development or recurrence of cancer (Friend & Shahani, 1984; Aso *et al*, 1995).

Consensus panels of experts on health attributes of lactic acid bacteria (Sanders, 1993; LABIP, 1995) concluded that

there were promising results related to positive effects of the consumption of lactic acid bacteria. Established benefits were identified on (a) lactose digestion, (b) several types of diarrhoeal diseases, (c) reduction of faecal enzymes that may play a role in colon cancer, and (d) the immune system. However, it was also concluded that additional research is necessary to confirm these beneficial effects in humans.

These literature data support the hypothesis that orally ingested *Lactobacillus casei* has specific health effects related to improvement of the composition and metabolic activity of the intestinal microflora and immunomodulation in humans. On the other hand, the probiotic strain should be safe for repeated human consumption in high numbers. Therefore, the objective of this strictly controlled study was to investigate the effect of consumption of a milk product fermented by *L. casei* strain Shirota (Yakult[®], Yakult Honsha Co. Ltd, Tokyo, Japan) in a Western type of diet in normal healthy subjects in terms of (a) the survival of the strain during passage through the gastrointestinal tract, (b) beneficial changes in the composition and metabolic activity of the intestinal microflora, (c) modulation of immune parameters, and (d) general health parameters and safety for human consumption.

Subjects and methods

Subjects

Twenty apparently healthy men, 55.8 ± 7.5 (SD) years of age were selected for this study. Inclusion criteria were no obvious obesity (BMI < 30 kg/m²), normal blood pressure (WHO criteria), no current medication affecting either the intestinal flora and/or the immune system and haematological and biochemical parameters. The study was performed according to the EU guidelines for Good Clinical Practice (GCP). Informed consent was obtained from all subjects, and the study was approved by the Institute External Medical-Ethical Committee.

Diet and design

During the 8-week study period, 20 subjects, randomly divided into a treatment group and a control group, received a strictly controlled diet with a constant composition of 2418 kcal (10 MJ), protein 11 en%, fat 28 en%, and carbohydrates 61 en%. The study consisted of stabilisation (2 weeks), test (4 weeks) and follow-up (2 weeks) periods. During the stabilisation and the follow-up periods, each subject consumed daily 3×100 ml sterilised semi-skimmed unfermented (Dutch) milk (1.5% fat). During the test period the treatment group received daily 3×100 ml *L. casei* Shirota-fermented milk containing 3.1% nonfat dry milk solids, 17% sucrose and flavours. The control group received the same volume of unfermented milk having a similar basic composition as the fermented product and packaged in identical bottles. Each batch of both products was checked at regular intervals for microbial composition. The fermented product contained at least 10^9 CFU *L. casei* Shirota per ml; the unfermented product was sterile.

The subjects were housed in the Metabolic Ward of the TNO Institute during the last three days of every fortnight, starting at the end of the stabilisation period. The subjects had their main meal at the institute each day and received the rest of the diet for the next 24 h period (breakfast, lunch, beverages, snacks and test or control drinks).

General health parameters

The following general health parameters were measured: body weight, body temperature, blood pressure, heart rate. Haematological parameters measured included white blood cell, red blood cell and platelet counts; haemoglobin concentration; haematocrit (Sysmex K1000-system); the sedimentation rate; and white blood cell differentiation. Biochemical parameters in serum measured included cholesterol, ASAT, ALAT, γ -GT, total protein, albumin, protein electrophoresis (albumin, α 1-, α 2-, β - and γ -globulins), C-reactive protein (CRP) and α 1-antitrypsin (α 1-AT).

Faecal microflora

Two grams of fresh faecal samples were collected from the inner part of the stool and were put immediately into pre-weighed bottles with 17 ml transport medium (TRM). The samples were weighed and stored at $4^\circ\text{C} \pm 1^\circ\text{C}$. Within 6 h the samples were homogenised in an anaerobic glove box, pipetted into four marked cryotubes (2 ml), and stored in liquid nitrogen. After thawing at 37°C in the anaerobic glove box, 10-fold successive dilutions were made in Peptone Physiological Saline. Aliquots of 0.1 ml of the appropriate dilution were spread onto the following agar media: Reinforced Clostridial Agar (Oxoid CM151) supplemented with 5 g/l glucose, 75 ml/l sterile horse blood and 75 ml/l (0.4%) China blue (RCB agar) for total anaerobic bacteria; RCB agar containing 80 mg/l kanamycin and 1 mg/l vancomycin for Bacteroidaceae; Eugon agar (BBL) supplemented with 10 g/l maltose (Merck), 400 ml vegetable juice (Campbell V8) and, after sterilisation, 5 ml/l sterile propionic acid to bring the pH at 6.0 ± 0.2 for *Bifidobacterium*. These culture media were incubated anaerobically in gas-tied plastic bags (Merck) at 37°C for 48 to 72 h.

Outside the anaerobic glove box, aliquots of 0.09 ml were spread by spiral plating (Spiral System Instruments, Bethesda, MD, USA) onto the following agar media: Rogosa agar (Oxoid) for *Lactobacillus*; LBS agar (Oxoid) containing 10 mg/l vancomycin and 2% lactitol for *L. casei* Shirota (large white colonies); Perfringens agar base (Oxoid) with 2 vials/l Perfringens SFP selective supplement (Oxoid) and 50 ml/l egg yolk emulsion for *Clostridium*; Baird-Parker agar (Oxoid) containing Egg yolk-Tellurite Emulsion for *Staphylococcus*; Slanetz and Bartley medium (Oxoid) for *Enterococcus*; Violet Red Bile Glucose agar (Oxoid) for Enterobacteriaceae, RCB agar containing 2 ml/l (1%) tellurite for *Bacillus*; Oxytetracycline-Glucose-Yeast Extract agar (Oxoid) with oxytetracycline GYE selective supplement for yeasts. These culture media were incubated anaerobically (GasPak) or aerobically at 37°C or 24°C . After incubation, the specific colonies on the selective culture media were counted and the number of viable microorganisms per gram faecal sample (CFU/g) were calculated. The mean and standard error per group were calculated from the log values of the CFU/g.

Bacterial enzyme activities

Faecal samples for the determination of β -glucosidase, β -glucuronidase, urease and tryptophanase were stored at -20°C until the assays were performed. β -Glucosidase activity was determined as follows. Substrate solution (2-nitrophenyl- β -D-glucopyranoside) was added to a homogenised suspension of faeces in phosphate-buffered saline (PBS) pH 6.5 (faecal dilution $\sim 1:100$). After incubation (20 min, 37°C) the enzyme reaction was stopped by the

addition of 0.01 mol/l NaOH. After centrifugation (10 min, 3000 × g), the *o*-nitrophenol formed was measured at 415 nm (Goldin & Gorbach, 1976).

For β -glucuronidase activity, substrate solution (phenolphthalein- β -glucuronide) was added to a homogenised suspension of faeces in PBS pH 6.5 (faecal dilution ~1:400). After 15 min incubation at 37°C the enzyme reaction was stopped by the addition of 0.2 mol/l glycerine solution (pH 10.4). After centrifugation (10 min, 3000 × g), the phenolphthalein formed was measured at 553 nm (Goldin & Gorbach, 1976).

The tryptophanase activity was measured in faecal samples diluted with phosphate-buffered saline (PBS, 0.05 mol/l, pH 7.0). To 1 ml diluted sample was added 2 ml cold acetone. The mixture was centrifuged and the supernatant was discarded. Then 1 ml PBS and 0.05 ml toluene were added. The samples were shaken (60 rpm) for 10 min. A pyridoxal-bovine serum albumin-PBS solution and substrate (tryptophan-PBS) was added to the samples. After 20 min of incubation (37°C), colour reagent (*p*-dimethylaminobenzaldehyde) was added. This mixture was incubated for 10 min at room temperature and centrifuged. The optical density at 540 nm was measured.

For the determination of urease activity, a test kit with a modified manufacturer's protocol was used (urea/ammonia test kit; Boehringer Mannheim, Mannheim, Germany). Urea and a buffer solution (triethanolamine pH 8.0) containing 2-oxoglutarate, glutamate dehydrogenase and NADH were added to a centrifuged (10 min, 3000g) faecal suspension. The amount of NADH oxidation was measured during 10 min at room temperature at 340 nm. All bacterial enzyme activities were expressed in terms of units (U) per 10¹⁰ CFU.

Faecal parameters

Faecal moisture content was derived from the difference between the faecal dry and wet weights. pH was measured in suspension of the pooled faecal samples.

Intestinal transit time was measured as follows. At arrival on the first day of each internal period, the subjects were given 500 mg carmine red. The time between ingestion and the first appearance of the red colour in the faeces was recorded and taken as the transit time. Neutral sterols (coprostanol, cholesterol, campesterol, β -sitosterol) and bile acids (cholic, lithocholic, deoxycholic, ursodeoxycholic and chenodeoxycholic acid) in faeces were measured by GLC according to the method of Child *et al* (1987).

Short-chain fatty acids (acetic, propionic and butyric) were analysed in faecal water by HPLC using a HPX 87-H column (30 cm × 7.8 mm, Biorad). Cytotoxicity of faecal water was assessed using a slightly modified version of the method described by Rafter *et al* (1987).

Urinary indices

Twenty-four-hour urine samples were collected during the periods when the subjects were housed in the metabolic ward. Spectrophotometric measurement of indican was performed using a colour reaction with thymol and FeCl (Gorter & DeGraaf, 1955). Urine was hydrolysed and phenol and *p*-cresol concentrations were determined by GLC with flame ionisation detection according to procedures of BCO laboratories (Breda, The Netherlands).

Immunology

Lymphocyte subsets: These were determined using fresh whole blood (K³EDTA) and double labelling procedures with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies (Becton Dickinson, 1989). The following combinations of monoclonal antibodies (Becton Dickinson, San Jose, CA, USA) were used: Leu3 FITC (CD4)/Leu2 PE (CD8) (T helper/inducer and T suppressor/cytotoxic cells); Leu4 FITC (CD3)/HLA-DR PE (T cells, activated T and B cells); Leu4 FITC (CD3)/Leu11 + 19 PE (CD16 + CD56) (T and NK cells); Leu18 FITC (CD45RA)/Leu3 PE (CD4) (T naive and T memory cells); Leu1 FITC (CD5)/Leu12 PE (CD19) (T and B cells, B cell subset); Leu4 FITC (CD3)/Leu12 PE (CD19) (T and B cells). Flow cytometric analysis was performed on a FACStar PLUS (Becton Dickinson, Mountain View, CA, USA).

Natural killer cell (NK) activity: NK activity was measured using mononuclear cells isolated from heparinised blood and ⁵¹Cr-labelled target (K562 tumour) cells (Mulé & Rosenberg, 1992). Using three different effector:target (E:T) ratios (100:1, 50:1 and 25:1) the lysis of target cells as represented by the subsequent release of ⁵¹Cr was determined as a measure of NK activity.

Cytokine assays: Interleukin 1 β (IL-1 β) and 2 (IL-2) and γ -interferon (IFN- γ) were measured in culture supernatants of stimulated (LPS 100 μ g/ml (Sigma, St Louis, MO, USA) for IL-1 β and ConA 20 μ g/ml (Sigma) for IL-2 and IFN γ) peripheral blood mononuclear cells (10⁶ cells/ml) using ELISA kits (IL-1 β and IL-2: R&D systems, Minneapolis, MN, USA; IFN γ : HBT, Leiden, The Netherlands).

Phagocyte functions: Flow cytometric analyses (FACS-can; Becton Dickinson) of phagocytic capacity and oxidative burst were done in fresh heparinised whole blood, using standard kits (Orpegen, Heidelberg, Germany).

Delayed-type hypersensitivity (DTH): To determine effects on the *in vivo* cellular response at week 9, the DTH reaction after 48 h against eight antigens (*Candida*, *Diphtheria*, *Proteus*, *Streptococcus*, tetanus, *Trichophyton*, tuberculin and glycerine (negative control) was tested using the Multitest CMI system (Institut Merieux, Lyon, France).

Humoral parameters: IgM, IgG, IgA, IgD and IgE and the complement factors C3, C4 and factor B were measured using a Behring Nephelometric Analyser (Behringwerke AG, Marburg, Germany).

Statistics

The statistical significance of differences in changes between groups was tested by using the non-parametric test of Sign-Wilcoxon. This test was performed after taking into account initial differences between treatment and control groups at the end of the stabilisation period.

Results

General health parameters

Throughout the study, there were no significant changes in general health parameters such as body weight, blood pressure, heart rate, temperature, haematology and blood chemistry in subjects of both the control and treatment groups.

Faecal microflora

During the test period, the consumption of *L. casei* Shirota-fermented milk resulted in a significant increase in the number of the administered *L. casei* Shirota ($P < 0.01$), reaching levels of 10^7 CFU per gram of wet faeces in the treatment group compared to the control group (Figure 1). Although not statistically significant, a concomitant increase in the total *Lactobacillus* count during the test period was observed (Table 1). In addition, in week 4 of the test period a significant increase in the *Bifidobacterium* count was observed in the treatment group as compared to the control group (Table 1; $P < 0.05$). The numbers of

Bacteroidaceae, Enterobacteriaceae, *Staphylococcus*, *Staphylococcus aureus*, *Bacillus*, *Clostridium*, *Enterococcus* and yeasts were not significantly different in the treatment group compared to the control group (Table 1).

Bacterial enzyme activities

Based on enzyme activities calculated per 10^{10} CFU, between-groups significant changes were observed at week 4 for β -glucuronidase (Table 2, Figure 2; $P < 0.05$) and β -glucosidase (Table 2, Figure 2; $P < 0.05$). Urease and tryptophanase activity showed no statistically significant changes.

Parameters in faeces and urine

Moisture content was significantly increased ($P < 0.05$) at the end of the test period (Table 2). Faecal pH was relatively stable throughout the study, varying from 7.0 to 6.8. No statistically significant effects were observed (Table 2). Intestinal transit time tended to decrease in both groups. This tendency persisted in the treatment group, resulting in a significant difference ($P < 0.05$)

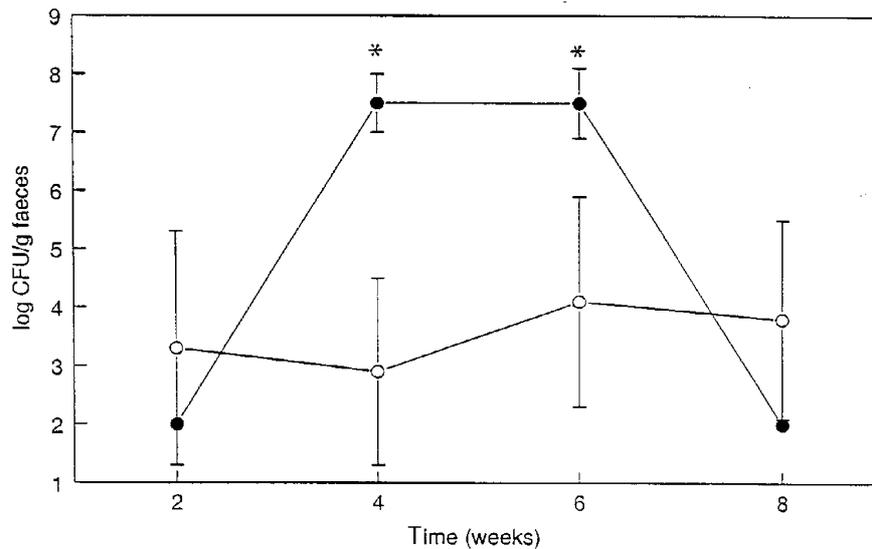


Figure 1 Mean numbers and s.e.m. (vertical bars) of *Lactobacillus casei* Shirota in faecal samples of the treatment (●) and control groups (○). * Significant difference between control and treatment group ($P < 0.01$).

Table 1 Log numbers of bacteria (mean \pm s.e.m.) per gram faecal sample measured in faecal samples at the end of the stabilisation period (week 2), after 2 and 4 weeks during the test period (week 4 and week 6), and at the end of the follow-up period (week 8)

Parameter	Control group				Treatment group			
	Week 2	Week 4	Week 6	Week 8	Week 2	Week 4	Week 6	Week 8
Total anaerobes	9.6 \pm 0.4	9.9 \pm 0.3	9.9 \pm 0.2	9.9 \pm 0.3	9.4 \pm 0.4	9.9 \pm 0.3	9.7 \pm 0.3	9.6 \pm 0.3
Bacteroidaceae	9.4 \pm 0.4	9.6 \pm 0.4	9.2 \pm 0.4	9.6 \pm 0.4	9.2 \pm 0.4	9.6 \pm 0.5	8.9 \pm 0.4	9.5 \pm 0.5
<i>Bifidobacterium</i>	9.1 \pm 0.3	9.1 \pm 0.6	9.3 \pm 0.4	9.3 \pm 0.5	8.8 \pm 0.5	9.2 \pm 0.5 ^a	9.2 \pm 0.4	8.9 \pm 0.6
<i>Lactobacillus casei</i> Shirota	3.3 \pm 2.1	2.9 \pm 1.8	4.1 \pm 1.8	3.8 \pm 1.9	2.0 \pm 0.0	7.5 \pm 0.5 ^a	7.5 \pm 0.6 ^a	2.0 \pm 0.0
<i>Lactobacillus</i> total	7.3 \pm 0.8	7.1 \pm 1.0	6.7 \pm 1.2	7.2 \pm 0.9	6.8 \pm 1.5	7.6 \pm 0.7	7.4 \pm 0.7	6.9 \pm 1.0
<i>Enterococcus</i>	6.2 \pm 0.8	5.6 \pm 1.2	5.7 \pm 0.9	5.2 \pm 1.3	5.5 \pm 0.8	4.7 \pm 1.1	4.3 \pm 1.5	4.3 \pm 1.4
<i>Clostridium</i>	4.6 \pm 1.6	4.5 \pm 1.0	3.6 \pm 1.8	3.3 \pm 2.5	5.2 \pm 1.0	4.7 \pm 1.0	3.3 \pm 2.0	3.7 \pm 2.2
<i>Bacillus</i>	3.1 \pm 1.1	3.1 \pm 1.1	2.6 \pm 0.3	2.8 \pm 1.1	2.9 \pm 1.1	3.6 \pm 0.8	3.0 \pm 0.5	3.5 \pm 0.4
<i>Staphylococcus</i> total	4.2 \pm 2.2	2.6 \pm 2.0	1.6 \pm 1.0	2.4 \pm 1.3	4.0 \pm 1.8	2.2 \pm 0.9	2.0 \pm 1.5	1.1 \pm 0.9
<i>Staphylococcus aureus</i>	1.0 \pm 0.2	1.2 \pm 1.0	0.8 \pm 0.1	1.0 \pm 0.8	1.2 \pm 1.2	0.9 \pm 0.3	1.1 \pm 1.0	0.9 \pm 0.6
Enterobacteriaceae	6.6 \pm 0.6	6.6 \pm 0.9	6.3 \pm 1.0	6.4 \pm 1.2	6.5 \pm 1.5	7.3 \pm 0.8	6.6 \pm 1.1	6.8 \pm 0.9
Yeast	1.9 \pm 0.9	2.2 \pm 1.3	2.1 \pm 1.0	1.6 \pm 1.2	1.5 \pm 0.8	1.8 \pm 1.1	1.4 \pm 1.2	1.2 \pm 1.1

^a Statistically significant difference ($P < 0.05$) between groups corrected for initial differences.

Table 2 Faecal and urinary parameters (mean \pm s.e.m.) measured at the end of the stabilisation period (week 2), after 2 and 4 weeks during the test period (week 4 and week 6), and at the end of the follow-up period (week 8)

Parameter	Control group				Treatment group				(Units)
	Week 2	Week 4	Week 6	Week 8	Week 2	Week 4	Week 6	Week 8	
Bacterial enzyme activities									
Urease	112 \pm 43	48 \pm 30	34 \pm 10	28 \pm 7	139 \pm 60	32 \pm 9	64 \pm 14	65 \pm 18	(10 ¹ U/10 ¹⁰ CFU)
β -Glucuronidase	80 \pm 20	45 \pm 7	41 \pm 6	55 \pm 14	167 \pm 35	44 \pm 6 ^a	72 \pm 13	123 \pm 24	(10 ⁻² U/10 ¹⁰ CFU)
β -Glucosidase	443 \pm 117	271 \pm 316	215 \pm 30	257 \pm 50	747 \pm 147	230 \pm 53 ^a	328 \pm 76	548 \pm 122	(10 ⁻² U/10 ¹⁰ CFU)
Tryptophanase	105 \pm 24	61 \pm 13	52 \pm 8	71 \pm 16	155 \pm 33	48 \pm 7	89 \pm 19	131 \pm 23	(U/10 ¹⁰ CFU)
Faecal parameters									
Faecal moisture	76 \pm 3	76 \pm 3	75 \pm 2	75 \pm 2	72 \pm 6	75 \pm 3	75 \pm 3 ^a	73 \pm 4	(%)
pH	6.9 \pm 0.2	6.6 \pm 0.4	6.8 \pm 0.3	6.8 \pm 0.4	7.0 \pm 0.4	6.9 \pm 0.2	6.9 \pm 0.2	7.0 \pm 0.3	
Intestinal transit time	45 \pm 14	30 \pm 16	36 \pm 15	37 \pm 15	44 \pm 14	35 \pm 11	29 \pm 12	26 \pm 18 ^a	(h)
Cytotoxicity of faecal water	9.6 \pm 3.2	11.5 \pm 4.3	12.6 \pm 4.0	8.8 \pm 2.3	13.6 \pm 5.2	10.3 \pm 2.3	12.4 \pm 4.4	8.8 \pm 3.0	(% lysis)
Coprostanol	60 \pm 37	42 \pm 18	59 \pm 36	66 \pm 46	72 \pm 30	68 \pm 19	64 \pm 20	66 \pm 20	(μ mol/g)
Cholesterol	10 \pm 17	10 \pm 12	6 \pm 4	6 \pm 4	6 \pm 3	5 \pm 3	4 \pm 2	5 \pm 2	(μ mol/g)
Campesterol	72 \pm 115	64 \pm 44	53 \pm 31	51 \pm 22	49 \pm 23	50 \pm 17	46 \pm 21	47 \pm 17	(10 ⁻² μ mol/g)
β -Sitosterol	18 \pm 22	20 \pm 15	12 \pm 8	14 \pm 8	14 \pm 8	14 \pm 7	13 \pm 6	13 \pm 6	(10 ⁻¹ μ mol/g)
Lithocholic acid	57 \pm 21	71 \pm 72	61 \pm 18	67 \pm 20	77 \pm 28	67 \pm 22	65 \pm 24	69 \pm 25	(10 ⁻¹ μ mol/g)
Desoxycholic acid	88 \pm 39	105 \pm 89	80 \pm 28	100 \pm 35	111 \pm 50	91 \pm 43	106 \pm 46	103 \pm 52	(10 ⁻¹ μ mol/g)
Chenodeoxycholic acid	13 \pm 19	16 \pm 23 ^a	14 \pm 25	16 \pm 19	8 \pm 6	6 \pm 5	6 \pm 3	6 \pm 3	(10 ⁻¹ μ mol/g)
Cholic acid	15 \pm 37	18 \pm 35	13 \pm 28	7 \pm 7	8 \pm 10	5 \pm 9	5 \pm 5	5 \pm 6	(10 ⁻¹ μ mol/g)
Ursodesoxycholic acid	35 \pm 62	33 \pm 54	58 \pm 99	51 \pm 94	30 \pm 64	24 \pm 39	13 \pm 15	16 \pm 18	(10 ⁻² μ mol/g)
Acetic acid	131 \pm 49	151 \pm 60	127 \pm 60	135 \pm 51	147 \pm 71	94 \pm 42 ^a	102 \pm 54 ^a	93 \pm 42 ^a	(mg/100ml)
Propionic acid	42 \pm 20	59 \pm 37	44 \pm 21	49 \pm 28	42 \pm 27	24 \pm 17 ^a	30 \pm 20 ^a	30 \pm 21	(mg/100ml)
Butyric acid	52 \pm 31	56 \pm 31	46 \pm 31	46 \pm 32	46 \pm 37	29 \pm 29	35 \pm 26	30 \pm 22	(mg/100ml)
Urinary indices									
Indican	39 \pm 15	38 \pm 10	44 \pm 13	38 \pm 10	46 \pm 15	47 \pm 16	44 \pm 14	43 \pm 13	(mg/ml)
Phenol	2.4 \pm 1.9	1.0 \pm 0.7	1.3 \pm 1.1	2.1 \pm 1.2	2.4 \pm 1.3	1.6 \pm 0.9	1.4 \pm 1.0	2.2 \pm 1.0	(mg/ml)
<i>P</i> -Cresol	49 \pm 31	38 \pm 30	57 \pm 27	39 \pm 28	62 \pm 29	70 \pm 44	57 \pm 26	52 \pm 24	(mg/ml)

^aStatistically significant difference ($P < 0.05$) between groups corrected for initial differences.

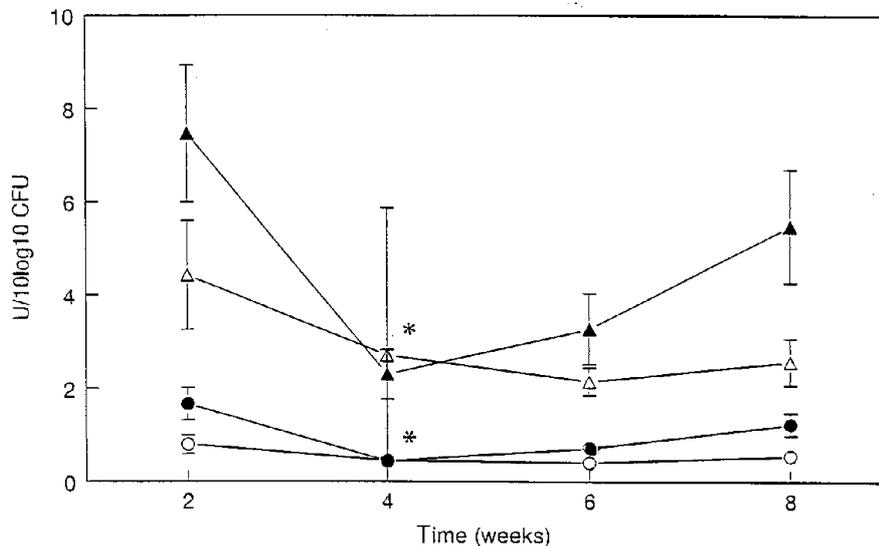


Figure 2 Mean β -glucuronidase (\circ , \bullet) and β -glucosidase (\triangle , \blacktriangle) activities and s.e.m. (vertical bars) in faecal samples of the treatment (solid markers) and control groups (open markers) calculated per 10¹⁰ CFU. * Significant difference in change of activity between control and treatment group ($P < 0.05$).

between the treatment and control groups at the end of the follow-up period (Table 2). Faecal concentrations (μ mol/g faeces dry weight) of neutral sterols and bile acids showed no significant differences (Table 2). All measured short-chain fatty acid (SFCA) (acetic, propionic and butyric) concentrations (mg/100 ml faecal water) showed similar trends, namely a decrease during the test period in the treatment group (Table 2). When compared between groups, these decreases were statistically significant for acetic acid at weeks 4, 6 and 8 and for propionic acid at

weeks 4 and 6 ($P < 0.01$). For butyric acid no statistically significant changes were found. Cytotoxicity of faecal water and urinary concentrations of indican, phenol and *P*-cresol showed no significant changes when treatment and control groups were compared (Table 2).

Immune system

No statistically significant effects were observed in the percentages of T cells, CD4⁺ cells, CD8⁺ cells, NK cells and B cells. Furthermore NK activity and production of

IFN- γ , IL-1 β and IL-2 showed no significant difference between the treatment and control groups. Similarly, there were no significant differences between the control and the treatment group in the humoral parameters measured (Table 3).

No statistically significant treatment effects were observed for phagocytic capacity, oxidative burst (Table 3) and DTH reactions.

Discussion

The study described in this paper is unique in that it is the first double-blind, placebo-controlled study with a commercially available probiotic product in healthy humans.

During the last 10 years it has been demonstrated in several studies that probiotic strains of lactobacilli, consumed via dairy products or given as freeze-dried preparations, may decrease the duration of diarrhoeal disease in children with intestinal infections (particularly with rotavirus) and in people with diarrhoea associated with antibiotic treatment (Siitonen *et al*, 1990; Isolauri *et al*, 1991, 1994; Kaila *et al*, 1992; Sheen *et al*, 1995). In addition, it has been demonstrated that probiotic lactobacilli may modulate parameters of the immune system (Perdigon *et al*, 1990; Sanders, 1993; Kaila *et al*, 1995; Pouwels *et al*, 1996). An important question, however, is what effect the consumption of probiotic lactobacilli has on intestinal ecology of healthy people. In spite of a rather large body of evidence in experimental animals, this question has not yet been answered, partly because of a lack of well-designed placebo-controlled experiments in healthy

humans (Marteau & Rambaud, 1993). In view of this, the present placebo-controlled study in healthy subjects was performed.

Regarding the general health of the subjects, the parameters measured, such as body weight, blood pressure and blood chemistry, did not reveal any significant changes, indicating that there were no adverse effects in either the treatment or the control group throughout the study.

The numbers of *L. casei* Shirota (Figure 1) recovered from the faeces confirmed the compliance of the subjects to the study protocol and demonstrated that an adequate percentage of *L. casei* Shirota survives passage through the gastrointestinal (GI) tract. Without exception, approximately 10^7 CFU of this strain per gram faeces were detected in all samples of the treatment group during the test period. After cessation of administration of the fermented milk, the numbers of *L. casei* Shirota returned to pre-treatment levels, indicating that this strain did not colonise the gut permanently. Similarly, another probiotic strain of *L. casei* (later characterised as *L. rhamnosus*) was found not to colonise the gut in several studies (Goldin *et al*, 1992; Saxelin *et al*, 1993, 1995). The average total number of *Lactobacillus* in the treatment group was not significantly different from that in the control group. However, in the treatment group the total *Lactobacillus* population in the faeces consisted to a large extent of *L. casei* Shirota.

The levels of faecal lactobacilli observed in the present study were high as those reported in previous studies (Hill *et al*, 1971; Yamagishi *et al*, 1974; Simon & Gorbach, 1984; Faassen *et al*, 1987; Mutai & Tanaka, 1987; Lidbeck,

Table 3 Immunological parameters (mean \pm s.e.m.) measured at the end of the stabilisation period (week 2), after 2 and 4 weeks during the test period (week 4 and week 6), and at the end of the follow-up period (week 8)

Parameter	Control group				Treatment group				(Units)
	Week 2	Week 4	Week 6	Week 8	Week 2	Week 4	Week 6	Week 8	
Lymphocyte subsets									
T helper (CD4)	45 \pm 8	46 \pm 9	48 \pm 9	47 \pm 9	44 \pm 8	45 \pm 6	47 \pm 6	45 \pm 7	(%)
T supp/cyt (CD8)	34 \pm 5	33 \pm 6	32 \pm 6	33 \pm 6	36 \pm 9	34 \pm 9	33 \pm 8	34 \pm 8	(%)
NK (CD16 & 56)	21 \pm 8	21 \pm 10	18 \pm 8	19 \pm 10	22 \pm 10	19 \pm 7	18 \pm 8	21 \pm 9	(%)
pan T (CD5)	70 \pm 12	69 \pm 12	72 \pm 11	71 \pm 12	70 \pm 10	72 \pm 8	72 \pm 7	70 \pm 9	(%)
pan B (CD19)	9 \pm 3	8 \pm 3	10 \pm 4	9 \pm 3	8 \pm 2	9 \pm 2	10 \pm 2	9 \pm 2	(%)
pan T (CD3)	70 \pm 11	69 \pm 11	71 \pm 10	71 \pm 12	70 \pm 10	71 \pm 7	71 \pm 7	69 \pm 8	(%)
Humoral parameters									
IgA	32 \pm 13	31 \pm 12	31 \pm 18	32 \pm 14	36 \pm 12	35 \pm 13	36 \pm 15	36 \pm 14	(10^{-1} g/l)
IgM	15 \pm 5	14 \pm 5	15 \pm 5	17 \pm 5	16 \pm 6	15 \pm 6	16 \pm 6	16 \pm 6	(10^{-1} g/l)
IgG	130 \pm 26	129 \pm 26	126 \pm 24	131 \pm 24	146 \pm 20	145 \pm 22	143 \pm 18	144 \pm 18	(10^{-1} g/l)
IgD	24 \pm 18	24 \pm 16	23 \pm 16	29 \pm 25	40 \pm 36	42 \pm 49	47 \pm 63	43 \pm 59	(U/ml)
IgE	42 \pm 32	44 \pm 34	42 \pm 33	40 \pm 29	87 \pm 76	85 \pm 81	85 \pm 83	85 \pm 82	(U/ml)
C3	82 \pm 10	81 \pm 11	84 \pm 14	84 \pm 13	83 \pm 13	82 \pm 13	78 \pm 11	80 \pm 17	(10^{-2} g/l)
C4	28 \pm 6	27 \pm 7	29 \pm 8	29 \pm 7	27 \pm 12	26 \pm 11	26 \pm 10	27 \pm 12	(10^{-2} g/l)
Factor B	178 \pm 41	173 \pm 38	185 \pm 45	181 \pm 44	189 \pm 35	182 \pm 38	182 \pm 33	188 \pm 43	(mg/l)
NK activity									
E:T ratio = 25:1	60 \pm 6	47 \pm 12	50 \pm 11	56 \pm 8	56 \pm 13	51 \pm 13	42 \pm 14	48 \pm 23	(% specific activity)
Cytokine assays^a									
IFN γ	176 \pm 99	138 \pm 71	193 \pm 123	193 \pm 106	117 \pm 48	113 \pm 62	108 \pm 94	99 \pm 53	(10 pg/ml)
IL-1 β	84 \pm 26	84 \pm 38	92 \pm 24	109 \pm 41	84 \pm 23	73 \pm 48	84 \pm 35	106 \pm 36	(10 pg/ml)
IL-2	60 \pm 30	58 \pm 33	50 \pm 21	63 \pm 40	40 \pm 28	48 \pm 31	46 \pm 22	49 \pm 29	(10 pg/ml)
Phagocyte functions^b									
Phagocytosis neutrophils	57 \pm 14	56 \pm 16	54 \pm 15	52 \pm 12	55 \pm 6	56 \pm 11	51 \pm 12	47 \pm 8	(%)
Oxidative burst neutrophils	19 \pm 9	24 \pm 6	22 \pm 8	16 \pm 7	20 \pm 8	21 \pm 7	19 \pm 4	15 \pm 9	(%)

^aFor IFN γ and IL-2 production mononuclear cells were stimulated with ConA 20 μ g/ml and for IL-1 β production with LPS 100 μ g/ml during 24h. ^bThe percentage of phagocytosing neutrophils was determined after 2.5 min incubation at 37°C; the percentage of neutrophils showing an oxidative burst was determined after stimulation with fMLP (5 μ mol/l) during 10 min at 37°C.

1991). The pre-existing high numbers of indigenous lactobacilli in the treatment group may have reduced the effects of *L. casei* Shirota administration on the total lactobacillus count. Nevertheless, the number of total *Lactobacillus* in the treatment group during the test period was higher than that at the end of the stabilisation and follow-up periods. This observation indicates that the consumption of a high number of *L. casei* Shirota increases the total lactobacilli count and does not simply replace the indigenous *Lactobacillus* flora.

The administration of *L. casei* Shirota was associated with a significant increase in *Bifidobacterium* counts, but did not have statistically significant effects on the numbers of the other microorganisms. It has been suggested that an increase in the *Bifidobacterium* count may indicate a beneficial effect on the stability of the intestinal flora (Mitsuoka, 1990). Since the faecal flora may not accurately reflect the microbial composition in other parts of the GI tract, we cannot exclude the possibility of more pronounced effects of *L. casei* Shirota administration on the microbial composition in specific parts of the ileum, caecum or colon. Synergistic effects of lactobacilli and bifidobacteria have also been observed *in vitro* in continuous cultures (Cheng & Nagasawa, 1983).

With respect to the metabolic activities of the intestinal flora, we observed a decrease in the β -glucuronidase and β -glucosidase activities, expressed per 10^{10} bacteria, upon administration of *L. casei* Shirota. Since these enzymes may be involved in chemical carcinogenesis (Goldin & Gorbach, 1984), this effect could be viewed as beneficial. Recent research in patients with superficial transitional cell carcinoma of the bladder indicates that oral administration of *L. casei* Shirota preparation (3 g per day) significantly reduced the recurrence of this disease after resection without side-effects (Aso *et al*, 1995). Although this observation is encouraging, further research is required to investigate the possible benefits of lower doses in healthy subjects, as used in the present study, before final conclusions can be drawn.

The observed increase in faecal moisture content (from 72% to 75%) in the treatment group, although small, may be of interest. We can only speculate about the underlying mechanism. It could reflect a decreased intestinal transit time and/or an osmotic intestinal effect. It is well recognised that the formation of short-chain fatty acids by the intestinal flora plays a role in water and electrolyte absorption and stimulates intestinal motility and osmotic pressure (Roberfroid *et al*, 1995). However, in contrast, we observed significantly decreased concentrations of short-chain fatty acids in the faecal samples of the treatment group. A reduced transit time may be responsible for the increase in the faecal moisture content, but the method used was not sensitive enough to detect small changes in intestinal transit time. A decrease in intestinal transit time has been recognised as preventing constipation and being protective with respect to colon cancer risk owing to an enhancement of the clearance of toxic compounds (Cumings *et al*, 1992).

No significant differences between the treatment and control groups were noted in the faecal excretion of neutral sterols and bile acids. Secondary bile acids, particularly deoxycholic acid, may have cytotoxic effects and increase epithelial cell proliferation and colon cancer risk (Jacobs, 1987). The lack of effects on faecal excretion of sterols, fatty acids and pH concurs with the absence of

effects on cytotoxicity of faecal water in the red blood cell lysis assay.

Studies in animals have demonstrated that oral administration of specific strains of lactobacilli may contribute to an enhancement of both the humoral and the cellular immune system (Havenaar & Spanhaak, 1994). Previous studies with healthy subjects that examined the effects of probiotics on the immune system were less well controlled and used high (3×10^{12}) or unreported amounts of lactobacilli per day (DeSimone *et al*, 1988; Halpern *et al*, 1991). In the present study no distinct effects on immune responses were noted during the consumption of *L. casei* Shirota-fermented milk. Although differences between the present study and those mentioned above, such as the *Lactobacillus* strain used, the dose level and the treatment period, could explain the lack of immune response effects in the present study, we think that other factors could also have played a role. One factor could be the above-mentioned masking effects by already high numbers of *Lactobacillus* in the intestine. Another factor could be that the selected healthy subjects had an optimal functioning and stable immune system in which clear-cut effects of consumption of fermented milk were not detectable. In contrast, Kaila *et al* (1992) observed the effect of a *L. casei* strain (later characterised as *L. rhamnosus*) on immune functions in rotavirus-infected children. Thus, it may be that, with respect to the effects of probiotic lactobacilli on the immune system, a distinction should be made between healthy, unchallenged subjects and individuals with a challenged (by infection or otherwise) or suppressed immune system. Further studies are needed to establish whether the administration of *L. casei* Shirota-fermented milk is able to induce effects on the immune system in immunosuppressed or immunocompromised individuals.

While the present study was performed with a rather limited number of subjects, it is worth noting that a similar study in Japan with the same product (Tanaka, 1996) showed almost analogous results to the study in the Netherlands, which supports the significance of the effects observed.

For some parameters a change over time was found in the treatment group as well as the control group. An influencing factor for this effect may be the short stabilisation period, which may have been too short for these parameters to reach a steady-state.

Test and reference products were identical with respect to their macronutrient composition; however, their pH values differed (3.5 versus 6.4 respectively). Although we cannot completely rule out that this pH difference influenced the results, we think this is unlikely. We are not aware of any data showing an effect of pH on any of the measured parameters.

We have demonstrated the survival of the ingested *L. casei* Shirota in the GI tract (Figure 1), which was associated with a small increase in the faecal *Bifidobacterium* count and a small reduction in activity of two bacterial enzymes (β -glucosidase and β -glucuronidase). We think that in healthy subjects with a normal, stable intestinal microflora, changes of larger magnitude would not be expected. It could be speculated that the changes observed may provide some additional defence mechanisms (improvement of mucosal gut barrier, colonisation resistance) in situations where the ecological intestinal balance is disturbed by penetration of enteropathogenic microorganisms. In addition, the formation of toxic compounds

may be influenced, which in the long term may reduce the cancer risk. At this moment, however, there is no direct evidence in humans for such beneficial effects.

Conclusions

In conclusion, this double-blind placebo-controlled study clearly demonstrates the survival of *L. casei* Shirota in the GI tract of adult healthy subjects. The consumption of *L. casei* Shirota was associated with some small, but statistically significant, changes in the composition and metabolic activity of the faecal microflora. Taking into account that the study was performed in healthy adult subjects and that large effects were not expected, these results could be meaningful. Further research is required to demonstrate the long-term significance of the observed changes for healthy individuals in terms of health maintenance or protection.

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