Eur J Nutr (2013) 52:1853–1863
DOI 10.1007/s00394-012-0487-1

ORIGINAL CONTRIBUTION

Immunomodulatory effects of a probiotic drink containing *Lactobacillus casei* Shirota in healthy older volunteers

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Received: 17 February 2012 / Accepted: 10 December 2012 / Published online: 10 January 2013

Abstract
Purpose There is growing evidence that probiotics confer health benefits to the host by modulating immune function, especially in older people, where immunosenescence is a feature even of healthy ageing. The aim of this study was to investigate the effect of a probiotic drink containing *Lactobacillus casei* Shirota (LcS) on immune function in a healthy non-immunocompromised older population.
Methods Thirty healthy old volunteers were recruited into a randomized placebo-controlled, single-blind cross-over study. The volunteers were supplemented with the probiotic drink containing 1.3×10^{10} CFU LcS or skimmed milk per day for 4 weeks, followed by 4 weeks of washout and were crossed over to the other treatment. Peripheral blood and saliva samples were collected at baseline and end of each treatment.
Results Probiotic consumption was associated with a significant increase in natural killer (NK) cell activity relative to baseline and a significant decrease in the mean fluorescence intensity of CD25 expression in the resting T cells compared with placebo. Additionally, there was a trend towards an increased ratio of IL-10 to IL-12 relative to baseline after LcS intake.
Conclusions Consumption of a probiotic drink containing LcS improved NK cell activity and tended to produce a more anti-inflammatory cytokine profile in an older population.

Keywords Cytokine · Immune function · *Lactobacillus casei* Shirota · Lymphocyte · Probiotic

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Introduction

The growth of the older population in most developed countries is set to continue to rise. Health issues faced by older people include a greater susceptibility to infection. It is well known that immune function becomes compromised with age, an effect known as immunosenescence [1]. Decreases in the numbers of T cells in peripheral blood, decreased capacity of T cells to proliferate and secrete cytokines, poor contribution to effective delayed-type hypersensitivity responses in vivo, a decline in innate immunity, including phagocytic and NK cell activity [2], and a decrease in specific antibody responses have all been reported in older people [3]. A poorly functioning immune system compromises the ability to respond to infections and to develop increased protection after vaccination, which contributes to a higher mortality rate in older people.

The human gastrointestinal tract supports a rich and complex microbiota, whose composition and activity play very important roles in nutrition, immunology and specific disease processes. It is reported that in older people, the numbers and species diversity of many beneficial or protective anaerobes, such as lactobacillus and bifidobacteria, are reduced [4]. This is particularly the case in antibiotic-treated elderly individuals, who also experience an increase in bacteria groups believed to be detrimental to health, such as facultative anaerobes, clostridia, enterobacteria, eubacteria and proteolytic bacteria [5]. Knowledge of the

composition of the elderly gut microbiota leads to an attractive means of dietary intervention by probiotic supplementation with immune-stimulating strains of lactic acid bacteria. Some human studies have reported that probiotic strains boost natural killer (NK) cell activity [6] and phagocytic activity [6, 7] in healthy volunteers and have beneficial effects in inflammatory and atopic diseases, possibly by modulating the production of pro- and anti-inflammatory cytokines [8]. There are very limited data, however, regarding the effect of probiotics on acquired immunity, in particular in older people.

Yakult is a fermented milk drink containing the probiotic *Lactobacillus casei* Shirota (LcS), which has been shown to survive digestion in the gut and reach the large intestine in a viable state [9]. Evidence from human studies suggests that LcS increases the number of beneficial intestinal bacteria, achieving a balance between beneficial and harmful intestinal bacteria [10], improves constipation [11], modulates immune function [12], alleviates the severity of symptoms in allergic rhinitis [13] and reduces risk of infection [14]. However, there are very few studies examining the effect of this probiotic on the immune function in the older population. The effects of probiotics on immune function extend to a wide range of parameters, with NK activity being one of the most commonly modulated. Each of the aspects of immunity evaluated in this study is subject to modulation by ageing; the hypothesis of this study was that LcS would at least partially reverse some of the decline in immune parameters caused by ageing. Of course, we cannot be sure that the elderly subjects who took part in this study were immunocompromised. However, immunosenescence is universally associated with ageing and is observed even in healthy ageing. The purpose of this study was not to select an immunocompromised group of subjects, but an older group that was expected to have some degree of immunosenescence. In this study, we assessed a wide range of immune parameters, since there are limitations associated with the evaluation and interpretation of single immune biomarkers.

Materials and methods

Subjects

Thirty healthy volunteers, 18 females and 12 males, 55–74 years old, were recruited for this study. The sample size was calculated by G* power software v 3.0.10 (Mahhneim, Germany), based on improved NK cell activity by *L. paracasei* (NCC 2461) supplement [2] with the effect size of 0.9, a two-sided significance level of 5 % and a power of 95 %. The inclusion criteria included 55–80 years old, body mass index (BMI) 19–30 kg/m^2 and good general health. The exclusion criteria were anaemia, milk allergy and lactose intolerance, any ongoing inflammatory or infectious disease, any autoimmune disease, malignancy, cirrhosis, use of anti-inflammatory or immunomodulating medication (including oral prednisone and other steroids) currently or within 4 weeks; use of antibiotics within 3 months, vaccination within 6 months and alcoholism and drug misuse. All volunteers signed an informed consent form and agreed to conform to the trial guidelines and provide notification of any non-compliance.

Study design

The study protocol was approved by the Research Ethics Committee at the University of Reading (Research Ethics Committee Project No. 09/34) and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All the volunteers gave their informed consent prior to their inclusion in the study. The study was designed as a randomized placebo-controlled, single-blind crossover study with a two week run-in period prior to the beginning of the study, when volunteers were required to refrain from any probiotic or prebiotic containing food or drinks and record a 4-day food diary, including one weekend day, to assess habitual diet. The volunteers were randomized by an Excel-based covariate adaptive randomization programme [15] to enter two intervention arms: probiotic (10 females, 6 males) or placebo (8 females, 6 males) stratified by age, gender and BMI. The placebo consisted of commercially available UHT skimmed milk in sterile containers labelled 'test milk', since its nutrient content very closely matches that of Yakult Light, but the subjects were unaware of the identity of the test milk. The volunteers were informed that the study was a comparison of the effects of the 'test milk' with Yakult on immune function. Therefore, the study was single-blind as the subjects were unaware that the 'test milk' was the placebo, or that only one of the products contained a probiotic. The subjects consumed 2 × 65 mL bottles of Yakult Light (commercially available) containing 6.5 × 10^9 CFU/bottle during the probiotic intervention period and skimmed milk (130 mL per day) during the placebo intervention period for 4 weeks on each arm, with a 4-week washout period. At baseline and at the end of each intervention period, overnight fasting venous blood samples were collected for immunological and biochemical analysis, and saliva was collected for sIgA measurement.

Preparation of PBMC

Fasted blood samples were taken from volunteers in sodium heparin vacutainer tubes (Greiner Bio-One Ltd, Gloucestershire, UK). Blood was layered over an equal

volume of Lympholyte (Cedarlane Laboratories Limited, Tyne & Wear, UK) and centrifuged at 930×*g* for 15 min at room temperature. The plasma was removed into a sterile tube for later use. Cells were harvested from the interface, washed once, resuspended in RPMI 1640 medium (Autogen Bioclear, Wiltshire, UK) containing 0.75 mM glutamine (Autogen Bioclear, Wiltshire, UK) and the above steps were then repeated to achieve a lower degree of erythrocyte contamination. The pellet was finally resuspended in RPMI, and the cell number was adjusted to the required concentration.

Preparation of other samples

Blood was collected by serum separator vacutainer tube (BD Biosciences, Oxford, UK) and centrifuged at 2,095×*g* for 10 min. Aliquots of serum were stored at −20 °C for later analysis of C5a, cholesterol, triacylglycerol, glucose and C-reactive protein (CRP). Saliva samples were collected and centrifuged at 13,000×*g* for 10 min. Aliquots of supernatant were stored at −20 °C for later analysis of sIgA.

Blood lipids, glucose and immune/inflammatory markers

The concentrations of serum cholesterol, triacylglycerol, glucose and C-reactive protein (CRP) were evaluated by iLab 600 (all kits and equipment from Instrumentation Laboratory, Warrington, UK). C5a in serum and sIgA in saliva samples were detected by commercial ELISA kits (C5a, BD Biosciences, Oxford, UK; sIgA, Oxford Biosystems, Oxford, UK), following the manufacturers' instructions.

White blood cell (WBC) count

Twenty microlitres of whole blood were diluted with 10 mL isoton (Beckman Coulter, High Wycombe, UK). Zapoglobin (Beckman Coulter, High Wycombe, UK) was added for 5 min, and WBCs were counted in the coulter (Coulter Z1 single 1.2.00, Beckman Coulter, High Wycombe, UK).

Phagocytic activity of leucocytes

Phagocytic activity of granulocytes and monocytes was determined using a commercial kit (ORPEGEN Pharma, Heidelberg, Germany). Heparinized blood was incubated with fluorescein-labelled opsonized *Escherichia coli* at 37 °C for 10 min. The reaction was stopped by addition of a quenching solution. After two wash steps, lysing solution was added and incubated for 20 min at room temperature, followed by another two wash steps before addition of DNA stain, propidium iodide (PI) (Sigma, Dorset, UK). The percentage of granulocytes or monocytes engaged in phagocytosis of *E. coli* and the mean fluorescence intensity (MFI) were detected by a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, Oxford, UK). Data were analysed using FlowJo 7.6 software (Tree Star, Inc., Ashland, USA).

Ex vivo culture conditions

PBMCs, adjusted to 2×10^6 cells/mL, were incubated in 24-well plates in the absence or presence of 1 μg/mL lipopolysaccharide (LPS) (L4516, Sigma, Dorset, UK) or 2.5 μg/mL concanavalin A (ConA) (Sigma, Dorset, UK) and 2.5 % autologous plasma for 24 h at 37 °C in an air/CO_2 (19:1) atmosphere. At the end of the incubation, Con A-stimulated cells were stained for activation markers, and supernatants from LPS-stimulated cells were collected and stored at −20 °C for later analysis of cytokine production.

NK cell activity

Freshly prepared PBMCs were adjusted to a concentration of 5×10^6 cells/mL. Viable target cells (K562 cell line) were enumerated by microscopy of trypan blue-stained cell preparations, and 5×10^6 cells were collected and washed twice with PBS before incubation with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (1 μg/mL) (Sigma, Dorset, UK) for 45 min at 37 °C in an air/CO_2 (19:1) atmosphere. After incubation, the target cells were washed twice and resuspended in 1 mL of complete medium composed of RPMI 1640 medium, 0.75 mM glutamine and 10 % newborn calf serum (Sigma, Dorset, UK). PBMCs were incubated with CFDA-SE labelled target cells for 2 h at 37 °C in an air/CO_2 (19:1) atmosphere at effector to target cell ratios of 100:1, 50:1, 25: 1 and 12.5: 1. Twenty microlitres of PI at 1 mg/mL were added to the samples prior to analysis by flow cytometry. The results were expressed as the percent lysis of the target cells. FlowJo 7.6 software (Tree Star, Inc., Ashland, USA) was used to perform the data analysis.

Lymphocyte phenotype and activation analysis

Cells were stained with appropriate combinations of fluorescently labelled mouse anti-human monoclonal antibodies for discrimination between different lymphocyte subsets. The expression of the activation markers, CD69 and CD25, on the following lymphocyte subsets was assessed: T lymphocytes ($CD3^+$), helper T cells (Th, $CD3^+CD4^+$), cytotoxic T cells (Tc, $CD3^+CD8^+$), NK cells ($CD3^-CD56^+$), $CD8^+$ NK cells ($CD8^+CD56^+$) and $CD8^-$ NK cells ($CD8^-CD56^+$). Monoclonal antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or (Cy-5 PE). PBMCs were incubated with fluorescently labelled monoclonal antibodies (all from BD

Biosciences, Oxford, UK) for 30 min at 4 °C in the dark and washed by Cell Wash (BD Biosciences, Oxford, UK) twice before fixing with Cell Fix (BD Biosciences, Oxford, UK). The fixed cells were analysed by flow cytometry within 24 h. The lymphocytes were gated, and fluorescence data for 10,000 events were collected and analysed by Flowjo 7.6. Results of phenotypes were expressed as the percentage of the subsets or within the cell group specified. Activation was determined as both the percentage of CD69 or CD25 positive cells in specific cell subsets and mean fluorescence intensity (MFI) for $CD69^+$ or $CD25^+$ cells within a particular cell subset.

Cytokine analysis in cell culture supernatants

The production of IL-1β, IL-6, IL-8, IL-10, IL-12 p70, IL-17A, IFN-γ, TNF-α, MCP-1, GM-CSF, MIP-1α, MIP-1β and RANTES was measured using Cytometric Bead Array (CBA) multiplex kits (BD Biosciences, Oxford, UK) by flow cytometry according to the manufacturer's instructions. BDTM CBA analysis software (BD Biosciences, Oxford, UK) was used to perform the data analysis.

T cell proliferation

T cell proliferation was measured by the Cell Trace CFSE Cell Proliferation Kit (Invitrogen Ltd, Paisley, UK). Briefly, PBMC cell suspensions at 1×10^6 cells/mL were incubated with CFSE (final concentration 0.01 mM) at 37 °C, 5 % CO_2 for 10 min and then staining was quenched by ice cold medium and the sample was incubated on ice for 5 min. After washing with RPMI medium containing 10 % autologous plasma, the cells were resuspended in the same medium and incubated in triplicate with either medium only (control) or with Con A at final concentrations of 15 μg/mL and 7.5 μg/mL at 37 °C, 5 % CO_2 for 4 days. The data were collected by flow cytometry and analysed using FlowJo 7.6 software.

Data are presented as mean and standard error (SE). All data were analysed using SPSS version 17.0. Significant differences between the probiotic and placebo periods and between baseline and end of intervention were evaluated by paired *t* test. The sequence effect was tested by indication of treatment*period interaction using two-way ANOVA, and no treatment*period interaction was observed. The carry-over effect of each treatment was evaluated by comparison of pre-treatment and post-washout. In one case, there was a carry-over effect of the Yakult treatment (% monocytes engaged in phagocytosis), and in this case, only the first period was analysed. Bonferroni multiple testing corrections were performed where applicable. The adjusted significant *P* value was calculated by the chosen level of significance *P* value (0.05) divided by the appropriate number of tests. Correlations between two normally distributed variables were analysed by using Pearson's correlation coefficients.

Results

All volunteers completed the study. There was 100 % compliance with the dietary intervention. The effects of the different interventions (probiotic or placebo) are detailed below.

Effects on blood lipids, inflammatory markers, complement and WBC count

There were no significant effects of probiotic on total cholesterol, triacylglycerol, glucose, CRP or C5a (data not shown). WBC count was significantly increased from 4.09×10^9/L to 4.54×10^9/L after the placebo treatment ($P < 0.05$), but was not significantly different from that during the LcS period.

Effects on salivary sIgA

Both LcS and placebo tended to increase salivary sIgA concentrations, but this did not reach statistical significance (data not shown). The probiotic had no effect on salivary sIgA in volunteers 65 years old or younger ($n = 18$), but there was a borderline increase in salivary sIgA ($P = 0.076$) in volunteers over 65y (data not shown) ($n = 12$).

Effects on phagocytosis

As there was a carry-over effect of Yakult treatment on the percentage of monocytes engaged in phagocytosis ($P = 0.000$), only the first period of the two treatments was analysed. There was no effect of placebo on phagocytosis compared with baseline. LcS significantly increased the percentage of monocytes engaged in phagocytosis ($P = 0.000$) and decreased MFI in granulocytes ($P = 0.002$) compared with baseline (Table 1); however, there was no significant difference between the two treatments.

Effects on NK cell activity

NK cell activity was significantly increased after intervention with LcS at an E/T ratio of 100 ($P = 0.008$), and almost reached significance for the E/T ratio of 50 ($P = 0.057$) compared to baseline (Fig. 1a). There was no effect of the placebo treatment on NK cell activity compared to baseline. Despite the increase in NK cell activity during the LcS intervention period, there were no

Table 1 Effect of probiotic LcS on leucocytic phagocytosis

	Placebo		Yakult	
	Before	After	Before	After
Granu (%)	95.49 ± 0.90	97.06 ± 0.30	94.09 ± 1.00	92.53 ± 2.43
Mono (%)	81.03 ± 1.91	84.90 ± 1.15	79.77 ± 1.59	87.50 ± 1.00[#a]
Granu MFI	268.56 ± 18.99	212.13 ± 18.20	277.52 ± 24.84	181.85 ± 16.53*
Mono MFI	130.17 ± 9.29	99.46 ± 8.26	132.53 ± 8.94	98.05 ± 9.53

Data are mean ± SE

The significant level of P value is 0.05/4 = 0.01 after Bonferroni correction

Granu granulocytes, *Mono* monocytes, *MFI* mean fluorescence intensity

* $P = 0.002$, # $P = 0.000$ compared with baseline

[a] Only the first period of the two treatments was analysed because of the carry-over effect of Yakult treatment

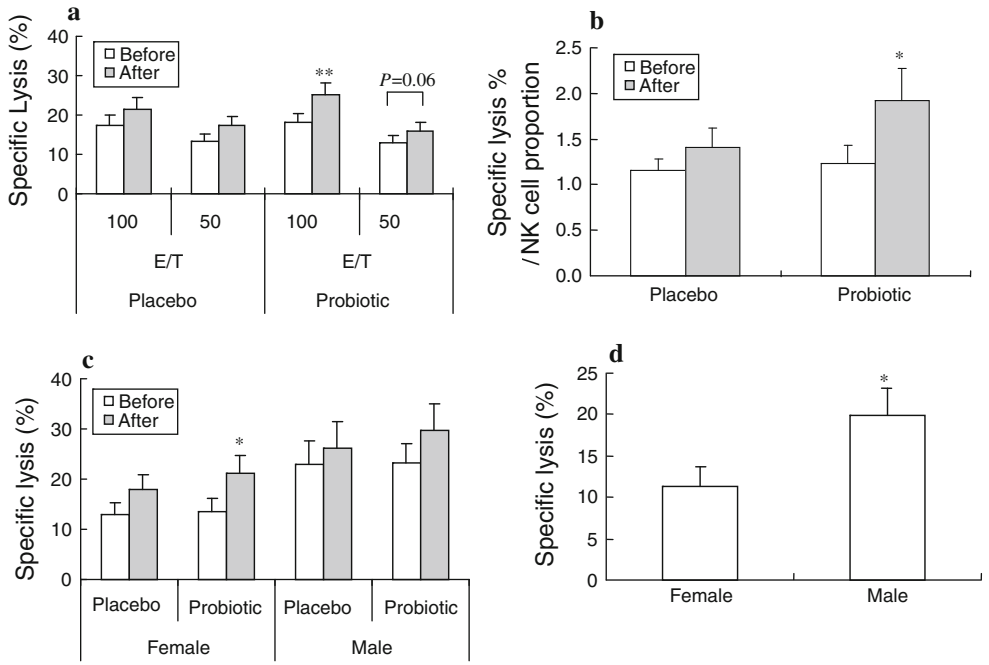

Fig. 1 Effect of probiotic LcS consumption on NK cell activity. Data are mean ± SE. **a** specific lysis (%) before and after probiotic (LcS) or placebo supplement. The ratios of effector/target cells (E/T) were 100/1 and 50/1. **b** Specific lysis on a per cell basis. E/T = 100/1. **c** Effect of LcS supplement on NK cell activity in female (n) = 18 and male volunteers ($n = 12$). E/T = 100/1. *$P < 0.05$, **$P < 0.01$ compared with baseline (before supplement). **d** NK cell activity at the beginning of the study in females and males. E/T = 100/1. *$P < 0.05$ compared with females

significant differences between probiotic and placebo periods at the end of intervention. Similarly, when NK cell activity was expressed on a per cell basis (divided by NK cell percentage in lymphocytes), LcS consumption was associated with significant increase in NK cell activity relative to baseline (Fig. 1b), but this was not significantly different from the placebo period. When gender was considered, the probiotic was shown to significantly increase NK activity compared with baseline in females ($n = 18$) ($P < 0.05$) but not in males ($n = 12$) (Fig. 1c). It should be noted, however, that NK cell activity at the beginning of the study was significantly higher in males (19.8 %) than in females (11.2 %) (Fig. 1d).

Correlation between NK cell activity and NK cell proportion and expression of activation markers on different subsets was analysed. NK cell activity showed strong correlation with the proportion of NK cells in the lymphocyte population ($P < 0.001$) and CD69 expression (both % and MFI) in the $CD8^-$ NK cells stimulated by ConA (both $P < 0.01$) (data not shown).

Phenotypic analysis of lymphocytes

A summary of lymphocyte phenotypes is shown in Table 2. There were no significant changes in lymphocyte phenotypes after probiotic supplement.

Effects on lymphocyte activation

The expression of two activation markers, CD69 and CD25, on specific lymphocyte subsets was assessed in both non-stimulated and Con A-stimulated cultures. LcS had no effect on CD69 expression in both of the resting (non-stimulated) (data not shown) and ConA-stimulated cells (Table 3) of all subgroups in comparison with placebo. Probiotic consumption was associated with significantly reduction in the MFI for CD25 expression by resting T cells ($P = 0.003$) relative to that during the period of placebo consumption (Table 4). There is no effect of LcS intake on CD25 expression on ConA-stimulated T cell subsets (Table 4).

Effects on cytokine production

The levels of a wide range of cytokines and chemokines were measured in supernatants from LPS-stimulated PBMC cultures. There was a tendency for IL-10 production to be reduced by the placebo treatment and increased by LcS, but this did not reach statistical significance (Table 5). Even though the baseline level of IL-10 in probiotic treatment was lower than that in placebo treatment, there was no significant difference in the basal values of IL-10 of both treatments ($P = 0.063$). The ratio of IL-10/IL-12 was marginally increased during the LcS period compared with the placebo period ($P = 0.009$) (Table 5).

Effects on T cell proliferation

Lymphocyte proliferation is expressed as Division Index and % Division. Division Index is the average number of cell divisions of responding cells, whereas % Divided is the percentage of the cells of the original sample which divided. There was no significant difference in either parameter between the probiotic and the placebo periods (data not shown).

Discussion

Some components of the immune response, including phagocytosis, NK cell activity and mucosal immunoglobulin A production (especially in children), have been shown to be improved by certain probiotic bacterial strains, while other components, including lymphocyte proliferation, the production of cytokines and production of antibodies other than immunoglobulin A, appear to be less sensitive to probiotic modulation [16]. The current human study has shown that LcS consumption was associated with an increase in NK cell activity, reduced MFI of CD25 expression on resting T cells and a trend towards an increased ratio of IL-10 to IL-12. There was no effect of LcS on T cell proliferation, phagocytosis, serum parameters, salivary sIgA or WBC count.

Immunoglobulin A (IgA) secretion in saliva decreases with age and may be related to increased susceptibility of

Table 2 Effect of probiotic LcS intervention on lymphocyte subpopulations

Group	Placebo						Probiotic (LcS)						*P* value[a]
	Before	SE	After	SE	Difference		Before	SE	After	SE	Difference		
					Mean	SE					Mean	SE	
T cell %/Lym	75.79	1.02	75.07	1.43	−0.72	0.81	74.26	1.40	74.35	1.23	−0.21	0.86	0.73
Th %/Lym	50.33	1.80	49.77	2.10	−0.56	0.65	49.25	1.94	51.45	2.15	1.15	0.89	0.05
Tc %/Lym	23.23	1.70	23.64	1.88	0.41	0.72	23.69	1.69	21.20	1.72	−1.77	0.79	0.11
Tc %/T cells	29.5	1.10	28.89	2.65	0.41	0.91	30.14	2.29	27.93	0.40	−2.21	1.03	0.07
NK %/Lym	13.99	0.98	14.22	1.11	0.23	0.82	15.78	1.19	15.00	1.16	−0.52	0.73	0.45
CD8 + NK %/Lym	14.35	1.07	16.06	1.21	1.71	0.93	17.66	1.06	14.77	1.43	−2.54	1.08	0.01
CD8-NK %/Lym	5.02	0.55	4.91	0.51	−0.11	0.25	5.16	0.56	5.32	0.58	0.29	0.20	0.22
CD8 + NK %/CD56+	76.38	1.51	77.87	1.47	1.49	1.13	79.50	1.40	74.30	1.84	−5.17	1.60	0.01
CD8-NK %/CD56+	23.21	1.50	21.34	1.42	−1.86	1.08	20.11	1.41	24.56	1.68	4.40	1.39	0.01

Data are mean ± SE

The significant *P* value after Bonferroni correction is 0.05/9 = 0.0056

Lym lymphocytes

[a] Paired *t* test on the mean change from baseline for the placebo and probiotic periods

Table 3 Effect of probiotic LcS intervention on expression of CD69 by ConA-stimulated lymphocyte subpopulations

Culture	Cell group	Parameters	Placebo						Probiotic (LcS)						*P* value[a]
			Before	SE	After	SE	Difference		Before	SE	After	SE	Difference		
							Mean	SE					Mean	SE	
ConA-stimulated	T cells	CD69 %	42.86	1.91	42.48	2.05	−0.37	1.55	38.60	1.77	41.62	2.25	3.02	1.76	0.11
		CD69 MFI	66.66	2.03	67.27	2.72	0.61	2.72	64.95	1.96	70.88	3.25	5.93	2.58	0.12
	Th	CD69 %	49.73	1.99	50.00	1.85	0.27	1.53	43.90	1.88	48.36	2.58	4.46	1.94	0.04
		CD69 MFI	85.79	3.65	85.09	3.85	−0.70	3.96	85.60	2.35	91.62	3.93	6.02	3.59	0.23
	Tc	CD69 %	40.82	2.40	38.97	2.98	−1.85	2.15	40.80	2.31	39.56	2.62	−1.24	1.95	0.84
		CD69 MFI	45.34	1.62	44.12	1.80	−1.22	1.66	44.73	1.30	47.83	2.95	3.10	2.43	0.08

Data are mean ± SE

The significant *P* value after Bonferroni correction is 0.05/6 = 0.0083

[a] Paired *t* test on the mean change from baseline between the placebo and LcS periods

Table 4 Effect of probiotic LcS intervention on expression of CD25 by lymphocyte subpopulations

Cultures	Cell group	Parameters	Placebo						Probiotic (LcS)						*P* value[a]
			Before	SE	After	SE	Difference		Before	SE	After	SE	Difference		
							Mean	SE					Mean	SE	
Unstimulated	T cells	CD25 %	7.83	0.48	6.63	0.44	−1.19	0.35	8.02	0.45	6.96	0.55	−1.20	0.48	0.99
		CD25 MFI	15.12	0.26	15.99	0.25	0.84	0.23	15.43	0.20	15.11	0.20	−0.22	0.29	0.003
	Th	CD25 %	9.36	0.56	8.91	0.68	−0.40	0.60	10.49	0.68	8.53	0.53	−2.15	0.76	0.06
		CD25 MFI	15.54	0.30	15.78	0.31	0.27	0.39	16.28	0.49	15.95	0.41	−0.21	0.29	0.41
	Tc	CD25 %	2.92	0.38	2.07	0.31	−0.86	0.32	3.78	0.48	2.69	0.39	−1.14	0.33	0.52
		CD25 MFI	14.48	0.55	14.72	0.37	0.23	0.70	13.48	0.28	13.93	0.38	0.37	0.50	0.87
ConA-stimulated	T cells	CD25 %	40.55	1.85	39.50	2.19	−1.05	1.31	36.82	2.01	39.30	1.95	2.48	1.70	0.09
		CD25 MFI	39.98	1.87	39.84	1.80	−0.14	1.39	38.01	1.93	38.82	1.71	0.80	1.51	0.70
	Th	CD25 %	46.02	2.05	44.92	2.08	−1.10	1.57	41.59	1.99	44.69	2.21	3.10	1.73	0.06
		CD25 MFI	37.92	1.20	35.57	1.49	−2.35	1.66	37.13	0.22	36.87	1.85	−0.26	1.75	0.44
	Tc	CD25 %	37.33	2.46	33.79	3.52	−3.53	1.96	35.15	2.60	33.30	2.81	−1.85	2.38	0.59
		CD25 MFI	55.36	2.95	52.97	3.44	−2.39	2.47	52.80	3.27	50.73	3.31	−2.07	2.86	0.95

Data are mean ± SE

The significant *P* value after Bonferroni correction is 0.05/6 = 0.0083

[a] Paired *t* test on the mean change from baseline between the placebo and Yakult periods

older people to respiratory infections [17]. Studies are inconsistent with regard to the influence of probiotics on sIgA [18–20]. The current study showed no effect of LcS on salivary sIgA secretion, but there was a marginal increase in total salivary sIgA secretion in volunteers over 65y. Since the number of subjects over 65y in the current study was rather small ($n = 12$), this marginal effect remains to be confirmed.

NK cells are critical for the removal of intracellular pathogens and also possess vital tumoricidal activities, but their activity declines with ageing [21], even though numbers of NK cells in the circulation increase with age [22]. Thus, NK cell activity is impaired when considered on a per-cell basis. NK cell activity has been demonstrated to be enhanced by the majority of probiotic strains studied in healthy adults [6, 7, 18] and in older subjects [23, 24], although a few studies report no effect [18, 25]. In the current study, LcS significantly increased NK cell activity both overall and on a per-cell basis. The ability of LcS to promote NK cell activity has also been reported in other animal/human studies [26–28]. In addition, LcS consumption was shown to reduce the recurrence of superficial bladder cancer [29], prevent atypia of colorectal tumours in a randomized trial [30] and reduce the risk of bladder cancer [31].

The current study showed that NK cell activity was higher in males than in females, which has been reported

Table 5 Effect of probiotic LcS intervention on cytokine/chemokine production by LPS-stimulated PBMC

Cytokine (pg/mL)	Placebo						Probiotic (LcS)						*P* value[a]
	Before	SE	After	SE	Difference		Before	SE	After	SE	Difference		
					Mean	SE					Mean	SE	
GM-CSF	114.16	15.74	117.94	18.25	3.78	15.06	101.01	16.21	83.93	10.25	−17.08	15.33	0.31
IFN-γ	33.05	13.89	37.52	9.13	4.47	12.16	87.25	62.04	49.10	19.84	−38.15	43.82	0.24
IL-10	343.42	69.84	314.75	49.08	−28.67	39.62	267.09	64.59	306.27	48.63	39.18	41.06	0.07
IL-12p70	5.06	0.62	5.28	0.62	0.23	0.48	5.50	0.65	5.42	0.55	−0.08	0.54	0.71
IL-17A	1.14	0.35	1.77	0.49	0.63	0.58	1.49	0.44	1.82	0.42	0.32	0.45	0.67
MCP-1	8,191.65	1,019.59	8,049.84	888.52	−141.82	640.07	8,584.31	966.53	8,340.17	849.95	−244.15	712.60	0.91
RANTES	2,907.01	187.04	3,413.50	260.09	506.49	268.86	3,241.55	224.22	3,375.84	299.88	134.28	276.95	0.32
TNF-α	426.17	76.63	380.14	60.75	−46.04	68.60	260.38	41.96	313.61	53.01	53.24	52.38	0.24
IL-1β	1,979.35	205.17	2,276.54	207.97	297.20	244.48	2,062.33	217.65	2,241.73	262.95	179.40	302.89	0.76
IL-6	15,220.78	1,190.89	15,432.74	891.24	211.96	1,283.10	15,584.70	1,281.72	14,169.52	944.53	−1,415.19	1,108.16	0.31
IL-8	66,231.30	3,866.58	67,623.92	3,131.52	1,392.62	3,991.48	68,757.59	4,117.72	64,173.38	3,390.92	−4,584.21	2,886.65	0.28
MIP-1α	5,599.89	440.81	5,573.80	334.21	−26.08	456.52	5,667.34	475.56	5,390.90	364.95	−276.44	399.72	0.69
MIP-1β	18,870.50	1,788.78	18,807.90	1,555.70	−62.60	1,838.64	18,263.86	1,214.79	17,930.37	1,549.01	−333.49	1,295.09	0.89
IL-10/IL-12	71.49	16.11	61.29	16.24	−10.20	9.24	46.02	6.95	71.63	14.80	26.74	13.70	0.009
TNF-α/IL-10	2.27	0.63	2.03	0.47	−0.24	0.40	1.68	0.46	1.46	0.35	−0.22	0.28	0.93
IL-1β/IL-12	463.01	63.30	593.74	118.85	130.74	102.54	478.08	71.88	581.05	110.39	103.13	113.95	0.97

Data are mean ± SE

The significant *P* value after Bonferroni correction is 0.05/13 = 0.0039

[a] Paired *t* test on the mean change from baseline between the placebo and probiotic periods

elsewhere [32]. It also demonstrated that LcS consumption had a greater effect on female than on male volunteers, and this effect might relate to the initially lower NK cell activity in females. This is supported by evidence that LcS enhanced NK cell activity in healthy younger subjects with relatively low NK cell [33], maintained NK activity in healthy older subjects [27] and restored NK cell activity in habitual smokers who had low NK activity [12]. It has thus been suggested that LcS does not necessarily enhance immune function above 'normal' levels in healthy individuals, but rather modulates it back to normal levels in situations where immunity is impaired, and thus plays a role in homoeostasis. However, this is contradicted by a study demonstrating no effect of LcS on NK cell activity in healthy subjects specifically selected for reduced NK cell activity [34].

Phagocytosis is decreased during ageing [35]. Several studies have demonstrated that various probiotic strains promote phagocytic activity in both healthy adults [7, 18, 19] and elderly subjects [24, 36], and one study reported that LcS restored phagocytic capacity back to normal in patients with alcoholic cirrhosis, while having no effect in healthy controls [37]. However, some studies report no effect on phagocytosis in colon cancer patients [38], healthy adults [39] and in-patients [40]. The current study showed that LcS consumption increased the percentage of monocytes engaged in phagocytic activity, but decreased granulocyte phagocytosis on a per cell basis. Therefore, the net effect of LcS on phagocytic capacity is not clear.

It is reported that age-related changes in human blood lymphocyte subpopulations include an increase in numbers and/or proportion of NK cells and $CD8^+$ T cells, but a decrease in T cells, $CD4^+$ T cells, B cells and the ratio of $CD4^+/CD8^+$ [41–43]. These changes in the T cell profile with age may result from increased susceptibility to apoptosis of T helper cells and greater resistance to apoptosis of cytotoxic T cells [44]. A combination of high $CD8^+$ and low $CD4^+$ and poor T cell proliferation in PBMCs was associated with higher 2-year mortality in a sample of very old Swedish individuals [45]. The data regarding the effect of probiotics on lymphocyte phenotypes are inconsistent. For example, an intervention study [25] with 122 healthy subjects (18–67 years) demonstrated that a mixture of three probiotic strains significantly increased the numbers of Tc cells, but had no effect on other lymphocyte subsets. Other studies demonstrated a significant increase in $CD3^+$, $CD25^+$ and $CD56^+$ cells, but no changes in $CD4^+$, $CD8^+$, $CD19^+$ or HLA-DR^+ cells after *B. lactis* HN019 intake in older volunteers [3] and an increase in $CD4^+$ T cell population, but no effect on other phenotypes by consumption of *L. paracasei* [46]. This increase in $CD4^+$ cells after probiotic consumption was also observed in mice fed *L. fermentum* PL9005 [47]. The current study showed that intervention with LcS had no effect on the proportion of Th or Tc cells. Consistent with this, there was no effect of LcS on the proportions of $CD4^+$ and $CD8^+$ cells in a small uncontrolled human study [27]. In addition, The current study demonstrated a decrease in CD25 expression by resting T cells and Th cells following LcS consumption. There is little comparative data, but similar results have been reported for *L. rhamnosus* GG [48].

The reported effects of probiotics on cytokine production are complex and suggest strain-specific differences [2, 27]. A range of cytokines and chemokines were examined in the current study, but there were no significant changes in cytokine and chemokine production by LcS. However, the ratio of IL-10 to IL-12 was tended to increase in association with LcS consumption, which indicates a shift towards a more anti-inflammatory profile. This could be beneficial to older people, given the increase in circulating inflammatory mediators with ageing as a result of lifelong infectious burden [42]. Other human studies with LcS showed no changes in stimulated cytokine production of IL-1β, IL-2, IL-4, IL-6, Il-8,IL-10, IFN-γ, TNF-α or MCP-1 in athletes [14], but significant reduction in allergen-induced IL-5, IL-6 and IFN-production compared with a placebo in volunteers with seasonal allergic rhinitis [13].

There are some limitations associated with the single-blind nature of the study, but this was unavoidable due to the nature and packaging of the products. The most appropriate placebo would ideally be a milk product identical to Yakult Light, but with no bacteria. This placebo has been employed in some studies [49, 50], but was unavailable to us. We selected UHT skimmed milk as the next best option, since its nutrient content very closely matches that of Yakult Light and it has been used as a placebo in several other trials [10, 27]. A further limitation was that we did not specifically select subjects who were immunocompromised. Each of the aspects of immunity evaluated in this study is subject to modulation by ageing, although because of the enormous variation amongst healthy subjects, defining thresholds for compromised immune function is very difficult. The hypothesis of this study was that LcS would at least partially reverse some of the decline in immune parameters caused by ageing, although it cannot be ascertained whether the subjects in the current study were immunocompromised. Immunosenescence is observed even in healthy ageing, and the purpose of this study was simply to investigate an older group (which was expected to have some degree of immunosenescence). It could be argued that the results from this study could also be applied to younger subjects and to those who are not necessarily immunocompromised. Finally, a limitation of all studies employing immune biomarkers in the absence of clinical outcomes is that it is

difficult to interpret the biological significance of minor immunomodulatory effects. There is a tendency to assume that because low NK activity is associated with higher risk of infection and cancer, reverting this will be beneficial. To date, there is little evidence to support this, and more clinical data are required.

In conclusion, the probiotic LcS may promote innate immunity by increasing NK cell activity and may improve inflammatory status by increasing the IL-10/IL-12 ratio in older people. Further studies are required to evaluate the immunomodulatory effects of LcS on the incidence and severity of infection, and the mechanisms by which LcS modulates immune function.

Acknowledgments All authors contributed to the study design, analysis of the data and preparation of the manuscript. All authors read and approved the final manuscript.

Conflict of interest This research was sponsored by a Dorothy Hodgkin Postgraduate Award and Yakult UK Limited. Dr. Thomas is an employee of Yakult UK Limited.

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