Immune response of healthy adults to the ingested probiotic *Lactobacillus casei* Shirota

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Abstract

Daily ingestion of a probiotic drink containing *Lactobacillus casei* Shirota (LcS; 1.3 x 10^{10} live cells) by healthy adults for (i) 4-weeks LcS, (ii) 6-weeks discontinuation of LcS, and (iii) a final 4-weeks of LcS, was investigated. There was a significant increase in expression of the T-cell activation marker CD3$^+$CD69$^+$ in *ex vivo* unstimulated blood cells at weeks 10 and 14 and a significant increase in the NK cell marker CD3$^+$CD16/56$^+$ in *ex vivo* unstimulated blood cells at weeks 4, 10 and 14. Expression of the NK cell activation marker CD16/56$^+$CD69$^+$ in *ex vivo* unstimulated blood cells was 62% higher at week 10 and 74% higher at week 14. Intracellular staining of IL-4 in *ex vivo* unstimulated and PMA/ionomycin-stimulated CD3$^+$ β7$^+$ integrin blood cells was significantly lower at week 10 and 14. Intracellular staining of IL-12 in *ex vivo* unstimulated and LPS-stimulated CD14$^+$ blood cells was significantly lower at weeks 4, 10 and 14. Intracellular staining of TNF-α in LPS-stimulated CD14$^+$ blood cells was significantly lower at weeks 4, 10 and 14. Mucosal salivary IFN-γ, IgA1 and IgA2 concentrations were significantly higher at week 14 but LcS did not affect systemic circulating influenza A-specific IgA or IgG and tetanus specific IgG antibody levels. In addition to the decrease in CD3$^+$β7$^+$ integrin cell IL-4 and a CD14$^+$ cell anti-inflammatory cytokine profile, at week 14 increased expression of activation markers on circulating T cells and NK cells and higher mucosal salivary IgA1 and IgA2 concentration indicated a secondary boosting effect of LcS.

Introduction

Intestinal bacteria interact with the host intestinal epithelial cells (including specialised M cells which pass antigen on to dendritic cells (DC) which lie below the epithelium) and gut-associated lymphoid tissue (GALT) both indirectly (via secretion of metabolic products and signalling molecules) [1], and by direct contact (including uptake by the GALT where DC process bacterial components and present them to lymphocytes) [2]. T-cells thus activated may either mount an active immune response to the bacteria or differentiate into Treg cells or Tr1 that suppress inflammatory responses [3-5]. Indigenous gut bacteria are also able to induce tolerance to their presence by induction of Treg cells or manipulation/antagonism of NF-κB, or through as yet unidentified mechanisms of T-cell hyporesponsiveness that promote gut homeostasis [3,6-8]. DCs appear to be one of the main gateways that determine the outcome of the above T cell responses. Communication between the microbiota and DC, and subsequent antigen presentation to naïve T-cells, is critically important. Different

bacteria induce different TLR signals, DC cytokine signatures and maturational trajectories by mechanisms not yet clearly defined [8,9,5,10-12]. Exactly how DCs discriminate between pathogenic and non-pathogenic bacteria is still not well understood although TLR responses, intestinal epithelial cells and cell wall molecular components on probiotic bacteria all appear to be involved (for example, in determining the cytokine responses of macrophages and DC) [8,13-16]. Certain probiotic strains have been found to reduce production of inflammatory cytokines and improve symptoms in inflammatory intestinal disease [17-20]. There is also evidence of probiotic improvement of IBS symptoms [21,22], but the data are sometimes conflicting and effects are considered to be strain-specific [23].

Trials with healthy subjects have shown that certain probiotic strains can increase intestinal secretory IgA (sIgA) and cytokine secretion. For example, intake of *Lactobacillus reuteri* ATCC 55730 by healthy adults was associated with an increase in epithelial B cells in the duodenum and CD4$^+$ T cells in the ileum [24]. A trial in healthy children observed increased total faecal IgA levels associated with *Bifidobacterium lactis* BB-12 intake [25]. Probiotic ingestion does not just increase IgA specific for the probiotic strain itself; increased secretion of IgA specific for polio [25] and influenza [26] vaccines has also been reported. This may be because IgA binding sites have a high level of cross-reactivity, so that an IgA molecule may recognise both a probiotic-derived epitope and a pathogen-derived epitope [27]. However, strains of both *Bifidobacterium bifidum* and *Lactobacillus casei* increased the total number of intestinal IgA-secreting cells in mice, without inducing production of probiotic- specific-IgA [28,29]. The mechanism behind this effect is not known but the implication is that some probiotics could alter general immune function without inducing a specific immune response.

The immune effects of probiotics are not limited to the intestine. Feeding of certain strains of *L. casei* to healthy adults has been observed to increase peripheral blood NK cell activity[30]; increase the oxidative burst capacity of monocytes [31]; and induce greater amounts of IFN-γ and TNF-α in whole blood when challenged with antigen [32]. Feeding of *L. casei* LC83F, for example, significantly increased salivary sIgA secretion in children [30] and feeding of *L. casei* Shirota (LcS) increased salivary IgA levels in athletes after prolonged exercise [33]. In addition, *Lactobacillus acidophilus* LAFTI®L10 increased salivary IFN-γ, but not IgA, in healthy athletes [34]. Furthermore, immune responses initiated at one mucosal site may be mirrored at others. Lymphocytes activated in the GALT travel in the blood before entering tissues as effector cells; they express specific chemokine receptors which allow them to preferentially re-enter mucosal tissues. Therefore,

immune responses initiated within the GALT can occur in the bronchus or lactating breast, and can be measured in secretions [35,36]. Nasal-associated lymphoid tissue (NALT), which includes the palatine and lingual tonsils located in the oral cavity, takes up antigen and activates immune cells in the same manner as the GALT [37,38].

Normal saliva contains a ratio of approximately 60:40 IgA1:IgA2 [39]. These two sub-classes of IgA differ in their structure at the hinge region. The hinge of IgA1 allows it to bind a broader range of epitopes than IgA2 however some bacteria and protozoans are able to secrete proteases that cleave IgA1 molecules. The IgA2 hinge region is more resistant to proteases [40] therefore IgA2 may be particularly important in the defence against such pathogens [41]. In a previous 4-week pilot feeding study with *L. casei* Shirota (LcS) in healthy volunteers, we found a significant increase in salivary sIgA secretion rate at 4 weeks and significant increases in salivary secretion rate of both IgA1 and IgA2 at 4 and 6 weeks [42]. It is therefore possible that probiotics such as LcS could reduce susceptibility to pathogens that enter the body via mucosal routes, such as the common cold, influenza or tuberculosis. There is in fact evidence that certain probiotic strains, including *L. casei,* may reduce the duration and/or incidence of respiratory infections [33,43-48]. The strain-specific nature of probiotic effects, which may be linked to their cell wall molecular characteristics and interaction with intestinal DC, is particularly apparent in studies where immune-modulation is a key mechanism of their activity, as illustrated by the differing outcome of two trials in athletes with different lactobacilli [33,49].

The current study was designed to investigate in healthy adult volunteers, the immunological effects of a commercial product containing the probiotic strain LcS, using a novel regimen comprising a primary (priming) 4-week ingestion period with the probiotic, then 6 weeks with no probiotic, followed by a further secondary (boosting) 4-week ingestion period with the probiotic. Using this regimen, data on the effects of intermittent LcS intake could also be investigated, because it is likely that in a societal setting, probiotic consumption could be intermittent. The following immunologic biomarkers were investigated at baseline (week 0) and at 4, 10 and 14 weeks: (i) peripheral blood intracellular cytokine (IFN-γ, IL-4, TNF-α, IL-12) staining in $CD3^+$ T cells (and those that traffic to the gut i.e. $CD3^+$, $\beta7^+$ integrin) and in $CD14^+$ monocytes; (ii) expression of NK ($CD3^+CD16/56^+CD69^+$) and T-cell ($CD3^+CD69^+$) activation markers; (iii) salivary IFN-γ, IgA1 and IgA2 and 4) specific IgA and IgG circulating antibodies to influenza A and tetanus.

Materials and Methods

Subjects

Subjects were recruited from the students and staff of the University of Greenwich at Medway UK, via a poster and an e-mail recruitment campaign. All potential volunteers were sent a Participant Information Sheet containing details of the study, along with a Consent Form and a 3-day food diary. Inclusion criteria were: age 18 – 49 years, healthy, with no chronic disease, not currently taking any medication, non-smokers, not drinking over 10 units of alcohol per day, not taking vitamin, mineral or fatty acid supplement, not pregnant or lactose intolerant. None of the subjects had taken the test probiotic before and there was exclusion of anyone who had taken any pro- or prebiotic products, consumed any other yoghurt or yoghurt drink more than 2-3 times per week, or had taken antibiotics within the previous two months. Volunteers who said they met the criteria and were willing to participate, attended an initial meeting where the exclusion criteria, study protocol and the food diary were explained and they were given the opportunity to ask questions. All subjects signed the Consent Form.

The probiotic tested was the commercial drink, Yakult Light, supplied by Yakult UK Limited. This food product is widely available and has been used in several trials in Europe. Each 65 ml bottle contained a minimum of 6.5×10^9 live cells of LcS at the end of the shelf-life. Fourteen (6 males, 8 females) subjects were asked to drink two 65 ml bottles (equivalent to a minimum intake of 1.3×10^{10} live LcS cells) daily with breakfast for 4 weeks, with a 6-week break between weeks 4 and 10, followed by resumption of probiotic ingestion for a further 4 weeks. Another four (2 males, 2 females) volunteers who met the study criteria but who did not consume the probiotic were used as controls for immune markers. A 3-day food diary (including two week days and one weekend day) was used to assess the food intake of each subject. Each subject was following a typical UK diet. Intake of vitamins A, C, E, zinc, iron, selenium and copper were all within the UK reference nutrient intake (DoH 1991) recommendations as were the macronutrients e.g. protein, carbohydrate, fat and energy intakes. The importance of drinking two bottles every day was stressed to the subjects at the beginning of the study. At each visit for blood and saliva collection, subjects were asked if they had had any problems with the probiotic drink and if they had taken it as requested. The study was approved by the University of Greenwich Research Ethics Committee (UREC/10/11.3.5.16).

Blood sample collection and preparation

Peripheral blood (~10 ml) from 18 healthy volunteers (including four volunteers used as immune marker controls) was obtained from the antecubital vein by venipuncture into 50 ml centrifuge tubes (Corning, USA) containing 7.5 mg (600 IU) of Heparin, sodium salt (Sigma, UK) diluted in 100µl PBS (Sigma, UK). Whole blood was transferred into 15 ml centrifuge tubes (Corning, USA) and used for immunofluorescence staining of surface molecules i.e. CD3, CD69 and CD16/56 (*ex vivo* unstimulated samples). Whole blood (~500 µl from each subject) was transferred into tissue culture test tubes (Bibby Sterilin Ltd., Stone, UK), diluted (1:1) in RPMI 1640 (Sigma, UK) containing 2 mM L-glutamine (Sigma, UK), 100 U Penicillin and 100 µg Streptomycin (Sigma, UK) and the 1 ml of diluted blood cultured in the presence of 1 µg ml^{-1} PHA (Sigma, UK) for 24 hours at 37 °C, 5% CO_2. Afterwards 250 µl of the diluted blood was used for surface immunofluorescence staining (i.e. CD3, CD69 and CD16/56, PHA-stimulated samples). Whole blood (3 ml from each subject) was transferred into 15 ml centrifuge tubes (Corning, USA), diluted 1:1 in RPMI 1640 supplemented with 100 U Penicillin and 100 µg Streptomycin, 2 mM glutamine, 1 mM pyruvate and 1 mM nonessential amino acids (Sigma, UK). 1.5 ml aliquots of diluted blood (5 x 10^6 leu ml^{-1}) were stimulated *ex vivo* (or *ex vivo* unstimulated) in tissue-culture multi-well (24) plates (Iwaki, Japan) either with 100 ng ml^{-1} LPS or with 3 µg ml^{-1} Phorbol 12-myristate 13-acetate (PMA) and 3 µM ionomycin, calcium salt (Sigma, UK) for 5 hours at 37 °C, 5% CO_2. Simultaneously, 3 µM Monensin was added to all 1.5 ml aliquots of the blood to inhibit cytokine secretion. Samples were used for intracellular staining of cytokines (i.e. IFN-γ, TNF-α, IL-4 and IL-12). The reminder of blood (5-6 ml from each subject) was centrifuged to remove plasma (800 g for 10 minutes) and finally plasma was centrifuged (800 g for 10 minutes), aliquoted into 1.5 ml centrifuge tubes and stored at -70°C for analysis of influenza A-specific IgA and IgG and tetanus specific IgG.

Saliva sample collection and preparation

Unstimulated saliva was collected over a five-minute period, by drooling and gentle pursing of lips into a sterile 50 ml centrifuge tube (Corning, USA). Subjects were asked to drink a glass of water 2 h before sample collection, to avoid food for one hour before collection and not to drink anything for ten minutes before collection. Subjects were asked to swallow once to clear the mouth, and then to sit quietly with head forward

to give the sample. Saliva samples were taken at baseline (week 0) and at weeks 2, 4, 10 and 14 of LcS consumption. Samples were collected at approximately the same time of the day, aliquoted and stored at -70 °C for IFN-γ, IgA1 and IgA2 analysis. The IgA1 or IgA2 secretion rate was calculated by multiplying the saliva flow rate (volume (ml)/5 min collection time) by the absolute IgA1 or IgA2 concentration in mg/ml.

Detection and quantification of salivary IFN-γ

Salivary IFN-γ levels were determined using a commercially available sandwich ELISA (enzyme-linked immunosorbent assay) system (Human High Sensitivity IFN-γ ELISA kit, Diaclone, UK) with a sensitivity of 0.78-25.0 pg ml^{-1}.

Detection and quantification of salivary IgA1 and IgA2

Salivary IgAl and IgA2 were measured using commercially available radial immunodiffusion assay kits (The Binding Site Ltd, UK). Agarose gel plates were supplied, which contained antibody mono-specific to either IgA1 or IgA2. The standard calibrator was human serum IgA1 and IgA2, at concentrations of 320 mg L^{-1} (IgA1) and 25mg L^{-1} (IgA2). The serum IgA1 and IgA2 controls were diluted by 20% and 60% and 10% dilutions were also made using 7% BSA. 5µl of standard or sample supernatant was pipetted into each well. After 30 minutes, a further 5µl of each sample was pipetted into each well in accordance with manufacturer's instructions for samples expected to contain low concentrations. All samples were used undiluted. The plates were sealed and incubated at room temperature for 96 hours, to allow equilibrium to develop between the formation and breakdown of antibody-IgA1 or IgA2 complexes. The complexes form a precipitin ring and at completion the diameter of the ring is proportional to the concentration of IgA1 or IgA2. The ring diameters were measured at completion to the nearest 0.1mm using digital callipers and the IgA1 and IgA2 concentration was read from a table provided with the manufacture's kit. Assay sensitivities were 640-6400 mg L^{-1} (IgA1) and 50-500 mg L^{-1} (IgA2).

Direct immunofluorescence staining for flow cytometry and flow cytometric analysis

For flow cytometry undiluted blood was transferred into tissue culture test tubes (Bibby Sterilin Ltd., Stone, UK) and in all experiments prior to incubation with antibodies, red cells were lysed using Human Erythrocyte Lysing Kit (R&D Systems, UK). Cells were stained with either 20 μl Human CD3 FITC (fluorescin labeled, Beckman Coulter, USA) and 20 μl Human CD69 PE (R-phycoerythrin labeled, Beckman Coulter, USA) or with 10 μl Human CD16/CD56FITC (Serotec, UK) and 20 μl Human CD69PE (Beckman Coulter, USA). 20 μl of Mouse IgG1 FITC (Beckman Coulter, USA) and 20 μl of Mouse IgG2b PE (Beckman Coulter, USA) were used as isotype controls and 100 μl of undiluted blood was used as an unstained control. CD69 (cell surface glycoprotein, activation marker) is undetectable on most of circulating PBMCs, however T-cells (CD3) and NK cells (CD16/56) express CD69 after activation with PHA. Cells were therefore stimulated with 1μg/ml of PHA. Diluted blood (250 μl) cultured in the presence of PHA (1μg ml^{-1}) for 24 hrs was transferred into tissue culture test tubes and stained with either 20 μl Human CD3 FITC (fluorescin-labelled, Beckman Coulter, USA) and 20 μl Human CD69 PE (R-phycoerythrin-labelled, Beckman Coulter, USA) or with 10 μl Human CD16/CD56FITC (Serotec, UK) and 20 μl Human CD69PE (Beckman Coulter, USA). Diluted blood (250 μl) cultured in the presence of PHA was stained with 20 μl of Mouse IgG1 FITC (Beckman Coulter, USA) and 20 μl of Mouse IgG2b PE (Beckman Coulter, USA) as isotype controls. 250 μl of diluted blood cultured with, and 250 μl without, PHA were used as unstained controls. Samples were mixed and incubated at room temperature in the dark for 15 to 20 minutes. The cells were then centrifuged (Labofuge, UK) for 5 minutes at 300 x g, supernatants were aspirated and cells were washed with 2 ml of working strength wash buffer (Human Erythrocyte Lysing Kit, R&D Systems, UK, 1:10 dilution wash buffer in distilled water). Afterwards the cells were mildly vortexed and centrifuged at 300 x g for 5 minutes. The supernatants were aspirated and discarded and the cells were re-suspended in 1 ml of working strength wash buffer. To stabilize the cells, 100 μl of Fixative buffer (Human Erythrocyte Lysing Kit, R&D Systems, UK) was added to 1 ml of cells re-suspended in wash buffer and the cells vortexed and stored at 2-8 °C until analysis within 24 h.

Intracellular staining of cytokines for flow cytometry

Fixaton of cells

After cell culture and stimulation of cells 1.5 ml aliquots were transferred into centrifuge tubes (15 ml, Corning, USA), diluted with 5 ml Hanks balanced salt solution (HBSS, Sigma, UK) and briefly vortexed. After centrifugation at 360 x g (Labofuge, UK) for 10 min at room temperature, supernatants were aspirated and discarded. 1 ml of 4% paraformaldehyde (PFA) was added and briefly vortexed. After 10 min incubation at 4 °C, tubes were filled again with HBSS, briefly vortexed and spun for 10 min at 360 x g. Supernatants were aspirated and discarded. The cell suspension was re-suspended in 1 ml of 5% non-fat dry milk (Sigma, UK) for 16 h at 4 °C in the dark to reduce nonspecific binding.

Permeabilisation of cells and antigen staining

After 16 h incubation at 4 °C and spun at 360 x g for 10 min at room temperature, supernatants were aspirated and discarded. 1 ml of 0.1% saponin buffer was added and vortexed briefly. 200 µl aliquots of this cell suspension were then transferred to centrifuge tubes (15 ml, Corning, USA) each containing 20 µl CD14 PE (Beckman Coulter, USA) and either 10 µl anti-human IL-12 FITC (eBioscience, USA) or 10 µl anti-human TNF-α FITC (eBioscience, USA) or each containing 20 µl CD3-PC5 (R-Phycoerythrin-Cyanine 5.1) and 10 µl anti-human integrin beta-7 PE (eBioscience, USA) and either 20 µl anti-human IFN-γ FITC (Beckman-Coulter, USA) or 20 µl anti-human IL-4 FITC (eBioscience, USA). After incubation for 20 min at 4 °C in the dark, 1 ml of 0.1% saponin buffer was added and vortexed briefly. Cells were spun at 270 x g for 5 min at room temperature and the supernatants were discarded. 0.5 ml of HBSS was added to all tubes, vortexed briefly and stored at 4 °C in the dark until analysis by flow cytometry within 24 h.

Flow Cytometry

Analysis of all samples was carried out using a flow cytometer (COULTER EPICS, XL·MCL, Beckman-Coulter, USA) fitted with a 488 nm argon-ion laser. Acquisition of data and subsequent analysis was performed with EXPO™32ADC software for Coulter EPICS XL cytometers (Beckman-Coulter). During flow cytometric analysis monocytes and lymphocytes were separated into two groups according to their size and granularity. An

area highlighting the lymphocytes and excluding the monocytes and debris was also generated. This was confirmed by expression of only CD3 in the lymphocyte gate. 100,000 events in this region were collected and analysed. Lymphocytes within this region were assessed for one of the pairs of the following cell surface molecules and/or cytokines: CD3 and CD69, CD3 and CD16/56, CD16/56 and CD69, β7 integrin and IL-4 and β7 integrin and IFN-γ. The expression of these antigens was detected using fluorescence-conjugated antibodies (FITC-CD3 and R-PE-CD69, FITC-CD3 and R-PE-CD16/56, FITC-CD16/56 and R-PE-CD69, R-PE-β7integrin and FITC-IFNγ, R-PE-β7integrin and FITC-IL-4). Isotype controls were, IgG1FITC, IgG1PC5 and IgG2b PE and IgG2aκPE. A region highlighting the monocytes and excluding the lymphocytes and debris was generated. Only CD14 was expressed in the monocyte gate, 100,000 events occurring in this region were collected and analysed. Monocytes present in this region were assessed for either pair of the cell surface markers and/or cytokines: CD14 and TNF-α or CD14 and IL-12. The expression of these antigens was detected using fluorescence conjugated specific antibodies (R-PE-CD14 and FITC-TNF-α, R-PE-CD14 and FITC-IFN-γ). Staining was determined by comparison to isotype controls (IgG1 FITC and PE-CD14).

Detection of influenza A-specific IgA and IgG

Influenza A specific IgA and IgG were determined using commercially available sandwich ELISA systems (IBL International, GMBH, Germany) coated with specific antigen. Each of these ELISA systems provided a 96-well microtiter plate, influenza IgA or IgG standards A-D (at 1, 10, 40 and 150 U/ml or 1, 10, 50 and 150 U ml^{-1}, respectively), anti-human IgA and IgG conjugated to peroxidase, buffers, TMB Substrate solution and TMB Stop solution. Each one of influenza IgA and IgG standards (100 μl) and diluted samples (100 μl, all samples were diluted 1: 50 according to the IBL instructions) were added in duplicate to the respective wells of the microtiter plate. The plate was covered and incubated for 60 min at room temperature. After washing (x3) with diluted wash buffer 100 μl of diluted enzyme conjugated anti-human IgA or IgG was added to all wells, covered and incubated for 30 min at room temperature. After the incubation each well was washed (x3) with diluted wash buffer and any remaining wash buffer was removed by blotting. Afterwards, 100 μl of TMB Substrate solution was added into each well and the plate was incubated for 20 min at room temperature in the dark. 100μl of TMB stop solution was added into each well and the absorbance at 450nm was read on a microplate reader (Original Multiskan Ex, ThermoLabsystems). A standard curve was constructed and the influenza A-specific IgA and IgG concentrations (U ml^{-1}) calculated using an equation generated by Excel. Sensitivity of both ELISA assays was 1 - 150 U ml^{-1}.

Detection of tetanus-specific IgG

Plasma tetanus-specific IgG was measured using a commercially available sandwich ELISA (IBL International, GMBH, Germany) coated with specific antigen. Anti-human IgG conjugated to peroxidase and IgG Standards A-E were at 0.0, 0.1, 1.0, 2.5 and 5.0 IU ml^{-1} were provided with the ELISA kit. The sensitivity of the assay was 0.0 to 5.0 IU ml^{-1}. The assay was performed as described for influenza A.

Statistics

The statistical package 'Excel' was used to calculate sample means and standard errors. Differences in the mean cell surface marker and intracellular cytokine staining between baseline and weeks 4, 10 and 14 of LcS consumption were analysed by ANOVA followed by post-hoc Paired two sample T-test and considered significant when P-values were <0.05. Differences between the means for each week were compared using Paired two sample T-test for salivary IgA1, IgA2, IFN-γ, influenza A-specific IgA, IgG and tetanus-specific IgG and considered significant when P-values were <0.05.

Results

The fourteen subjects (all LcS-naïve) who completed the intervention with the probiotic drink reported no noticeable adverse effects. Two reported improved stool consistency and two reported improved perception of health. One subject withdrew from the study at week 8 due to an aversion to blood collection. All subjects stated that they had maintained full compliance throughout the study. Ten subjects stated that the probiotic drink tasted pleasant, one expressed a mild dislike and four were not sure whether they liked it or not. The sensory perception did not interfere with compliance.

Effects on salivary IgA1, IgA2 and IFN-γ

Salivary IgA1 and IgA2 concentration

Mean salivary IgA1 concentrations were obtained on 10 of the 14 subjects. In all of them, the mean salivary IgA1 concentration was greater than baseline at weeks 2, 4, 10, however this was not significant. The increase in mean IgA1 levels was, however, statistically significant at week 14 of LcS ingestion compared with baseline (*P*=0.028, Figure 1). Mean salivary IgA2 levels were greater than baseline at weeks 2, 4 and 10, although this increase was not statistically significant. The mean IgA2 concentration was higher and statistically significant, compared with the baseline week 0 concentration, at week 14 (*P*=0.033, Figure 2).

Salivary IgA1 and IgA2 secretion rates, corrected for saliva flow rate

The IgA1 or IgA2 secretion rate was calculated by multiplying the saliva flow rate (volume (ml)/ 5 min collection time) by the absolute IgA1 or IgA2 concentration in mg/ml. There was an increase in mean IgA1 secretion rate at week 2, 4 and 10 however this was not statistically significant. Mean IgA1 secretion rate was significantly increased at week 14 (*P*=0.027, Fig.3). There was also a statistically significant increase in mean IgA2 secretion rate at week 14 (*P*=0.047, Fig.4). The mean IgA2 secretion rate was also increased at weeks 4 and 10, however this increase was not statistically significant.

Salivary IFN-γ

We measured salivary IFN-γ from 12 of the 14 subjects (data not shown). Several of the samples had IFN-γ concentrations below the detectable level of the assay. Six of twelve subjects (50% of the samples) had detectable IFN-γ levels at baseline, seven (58% of the samples) at week two, seven (58% of the samples) at week four, six (50% of the samples) at week ten and seven (58% of the samples) at week fourteen. Seven (58%) out of twelve subjects showed an increase in IFN-γ levels at week four and at week fourteen. Six (50%) subjects had detectable salivary concentration of IFN-γ throughout the study (weeks 0, 2, 4, 10 and 14), one had detectable salivary levels of IFN-γ only at week two and four, and one at week fourteen. The salivary

levels of IFN-γ at baseline were significantly lower than those at week 14 ($P<0.05$) of LcS ingestion.

Effects on human T-cell (CD3$^+$) and NK cell (CD16/56$^+$) cell surface marker expression

The mean cell surface expression of CD3$^+$CD69$^+$ (*ex vivo* unstimulated) was significantly higher at weeks 10 ($P=0.021$) and 14 ($P=0.0048$) of LcS feeding (Table 1) compared with the baseline week 0 values. In contrast, the mean cell surface expression of CD3$^+$CD69$^+$ (*ex vivo* PHA-stimulated) was significantly lower at weeks 4 ($P=0.027$), 10 ($P=0.027$) and 14 ($P=0.021$) of LcS ingestion (Table 1) compared with the baseline week 0. The mean cell surface expression of CD16/56$^+$CD69$^+$ (*ex vivo* unstimulated) was 11%, 62% and 74% higher at weeks 4, 10 and 14 respectively of LcS ingestion in healthy volunteers compared with that of the baseline week 0 value, however this was not statistically significant (Table 1). The mean cell surface expression of CD16/56$^+$CD69$^+$ (*ex vivo* PHA-stimulated) showed no statistical significance at weeks 4, 10 and 14 of LcS ingestion compared with the baseline week 0 values. The cell surface expression of CD3$^+$CD16/56$^+$ (*ex vivo* unstimulated) in healthy volunteers was significantly higher at weeks 4 ($P=0.043$), 10 ($P=0.0049$) and 14 ($P=0.0004$) of LcS ingestion compared with baseline week 0 (Table 1). The mean cell surface expression of CD3$^+$CD16/56$^+$ (*ex vivo* PHA-stimulated) was 31%, 56% and 43% higher than week 0 at weeks 4, 10 and 14 respectively of LcS ingestion although this was not statistically significant.

Effects on intracellular cytokine staining

The mean intracellular staining of IFN-γ in *ex vivo* unstimulated and PMA and ionomycin stimulated CD3$^+$β7$^+$ integrin cells showed no statistical significance between weeks 0, 4, 10 and 14. The mean intracellular staining of CD3$^+$β7$^+$ integrin cell IL-4 in PMA and ionomycin-stimulated cells was significantly lower ($P=0.037$) at week 14 of LcS ingestion compared with the baseline week 0 (Table 2.). The mean intracellular staining of CD3$^+$β7$^+$ integrin cell IL-4 in *ex vivo* unstimulated cells was significantly decreased ($P=0.034$) at weeks 10 and 14 of LcS ingestion compared with the baseline value (Table 2). The mean intracellular staining of IL-12 in *ex vivo* LPS-stimulated and *ex vivo* unstimulated CD14$^+$ cells was significantly lower at week 4 ($P=0.0008$) week 10 ($P=0.0013$) and week 14 ($P=0.00003$) and at weeks 4 ($P=0.005$), 10 ($P=0.0034$) and 14 ($P=00062$), respectively, of LcS ingestion compared with the baseline week 0 values (Table 2). The mean intracellular staining of TNF-α in *ex vivo* LPS-stimulated CD14$^+$ cells was significantly lower at weeks 4

(P=0.0003), 10 (P=0.0058) and 14 (P=0.032) of LcS ingestion than that of baseline (Table 2). Intracellular staining of TNF-α in *ex vivo* unstimulated CD14$^+$ cells showed no statistically significant differences between LcS ingestion and baseline week 0 (Table 2).

Influenza A-specific IgA, IgG and tetanus-specific IgG

Influenza A-specific IgA in plasma was analysed using an ELISA assay for the 14 subjects consuming LcS. Seven of the fourteen samples (50%) showed positive levels at weeks 0, 4, 10 and 14 of LcS ingestion. There were no significant or apparent individual differences in influenza A-specific IgA between baseline and weeks 4, 10 and 14 of LcS ingestion (Table 3.). Only those subjects showing positive results for influenza A- specific IgA levels were selected for the plasma influenza A-specific IgG assay (i.e. 7 subjects from 14). Five out of seven samples (71%) showed positive levels at baseline week 0 and weeks 4, 10 and 14 of LcS ingestion. There was no significant difference in influenza A-specific IgG between baseline and weeks 4, 10 and 14 of LcS ingestion (Table 3). Seven subjects consuming LcS were selected for the plasma tetanus specific IgG assay. Five out of seven samples (71%) showed positive levels at baseline week 0 and weeks 4, 10 and 14 of LcS ingestion. There was no statistically significant or apparent individual association between tetanus specific IgG and LcS ingestion at any of the time points sampled (Table 3).

Discussion

In this study, ingestion by healthy adult volunteers of LcS at a dose of 1.3 x 10^{10} live bacteria per day for 14 weeks, with discontinuation of the probiotic between weeks 4 and 10, resulted in significantly higher expression of the T-cell activation marker CD3$^+$CD69$^+$ and 62% and 74% increase in the NK cell activation marker CD16/56$^+$CD69$^+$ in peripheral blood cells (*ex vivo* unstimulated) at weeks 10 and 14 respectively. CD69 has well known receptor stimulatory functions on T cell and NK cell proliferation, lymphokine (s) secretion and cytolytic activity and also regulates other NK cell functions such as CD25 and ICAM-1 expression [50]. We observed a significant increase in the NK cell marker CD3$^+$CD16/56$^+$ (*ex vivo* unstimulated) at 4, 10 and 14 weeks. The increase in cell activation markers at week 10 show that after discontinuation of LcS there is

maintenance of the activation state of these cells for at least 6 weeks. Furthermore, the increase in some of the above immunologic markers at 14 weeks indicates that there was a secondary boosting effect of the LcS feeding regimen. These findings are in general agreement with those of Dong et al [51] who demonstrated significant increases in the expression of the activation marker CD69 on NK cells and T cells (CD8 and CD4) in human peripheral blood mononuclear cells (PBMC) co-cultured *in vitro* with LcS. They are also consistent with the reported increase in or maintenance of functional NK cell activity in other subjects consuming LcS: healthy office workers [46], middle-aged and elderly people [52,53], and smokers. In the latter study, LcS intake increased the low NK cell cytotoxic activity of the smokers, which correlated with an increase in CD16$^+$ cells [54]. Several animal studies have also shown increased NK cell activity following LcS or other probiotic *Lactobacillus* feeding, both with and without concurrent infection [55-59]. Seifert et al [60] did not find significant effects of LcS feeding on human CD3$^+$CD56$^+$ expression and NK cell function, possibly because they did not use CD16 in their immune-phenotyping panel and subjects were pre-selected for low NK cell lytic activity. Our findings have significance for immunosurveillance, for example NK cells are important in physiological immune surveillance e.g. lysis of virally infected cells and tumour cells, and low human NK cell activity has been linked to increased cancer risk [61]. Furthermore, human studies indicate an association between LcS ingestion and protection against various cancers [62].

We observed a significant decrease in intracellular staining of IL-4 in *ex vivo* unstimulated and also in PMA- and ionomycin-stimulated CD3$^+$β7$^+$ integrin cells at weeks 10 and 14 of LcS ingestion with no difference in Th1 pro-inflammatory IFN-γ intracellular staining. This is the first report of decreased intracellular IL-4 staining in CD3$^+$β7$^+$ integrin cells with LcS ingestion and may have important implications for Th1/Th2 deviation within the gut compartment and Th2 IgE immune-mediated intestinal inflammation. These cells are known to home to the gut via the binding ligand MAdCAM-1, expressed in the gut, and are involved in inflammatory intestinal disease.[63-65] Lactobacilli and bifidobacteria have been reported to reduce Th2 cell IL-4 and IL-5 production in animal studies and in the serum/PBMC from healthy subjects and patients with allergy and appear beneficial in IgE associated eczema in infants [66-69]. LcS could have potential beneficial effects in allergic conditions [70] and in particular food allergy. Studies on the effects of LcS and other probiotics on the ratio of allergen-specific IgE antibody to antibodies of blocking isotypes i.e. IgG4 and in particular IgA are warranted.

We found a significant decrease in the intracellular staining of pro-inflammatory IL-12 in *ex vivo* unstimulated and LPS-stimulated CD14^{+} monocytes and a decrease in pro-inflammatory TNF-α intracellular staining in LPS-stimulated CD14^{+} monocytes at weeks 4, 10 and 14 of LcS feeding. The reduction of CD14^{+} intracellular TNF-α staining is consistent with other human and animal studies showing *Lactobacillus* probiotics decrease TNF-α [71,20,72-74] although not all [51]. It is possible that these are some of the strategies employed by LcS to avoid host defence mechanisms. Our findings of reduced systemic circulating CD14^{+} intracellular IL-12 staining by LcS feeding are not, however, in agreement with those reported for *Lactobacillus* and other probiotics using different biological systems and detection methods to study this cytokine. It is generally considered that *Lactobacillus* probiotics increase IL-12 production [75-77,13,15,16,10,78,79] but there are reports that *Lactobacillus* strains at high doses reduce IL-12 via the induction of the immunoregulatory and anti-inflammatory cytokine IL-10 and that LcS-induced IL-12 production may be modulated by other bacteria and their cell wall components [78,80]. Moreover, some of the studies showing increased IL-12 have been carried out *in vitro* or *ex vivo* and therefore may not fully reflect the complex interactions that occur *in vivo* in relation to IL-12. In addition, studies have been conducted in inbred strains of experimental animals that do not always reflect the findings of human studies. IL-12 is known to drive IFN-γ and the Th1 response, but interestingly increases in IL-12 can decrease Treg function and Foxp3 levels in Tregs [81]. It has also been reported that some probiotics can increase Tregs and induce the anti-inflammatory and Treg cytokine TGF-β [82]. This cytokine and also IL-10 have been shown to have a regulatory effect on IL-12 and a reciprocal TGF-β and IFN-γ response regulates the occurrence of mucosal inflammation [83-85]. Thus the overall balance between the later cytokines as well as Treg development induced by a probiotic is likely to be important. Furthermore, LcS and other probiotics may encourage a macrophage anti-inflammatory M2 phenotype e.g. TGF-β and IL-10 rather than an M1 pro-inflammatory phenotype e.g. TNF-α, IL-1, IL-12 and in this way reduce direct macrophage mediated inflammation and modulate Th1 and Th2 cell mediated inflammation. Additional support for the above hypothesis comes from Habil et al [86] who reported LcS differentially affects M2 macrophage IL-6 and NFκB.

LcS ingestion resulted in significantly higher levels of mucosal salivary IFN-γ as well as significantly increased mucosal salivary IgA1 and IgA2 concentrations at week 14. The increase in IgA1 and IgA2 at week 14 appears to indicate that the scheduling of LcS had a secondary boosting effect. These findings are broadly consistent with our earlier report where we observed a transient increase in mucosal salivary IFN-γ at week 2 and increased IgA1 and IgA2 concentrations at weeks 4 and 6 (2 weeks after cessation of LcS feeding) of a 4

week LcS feeding study [42]. Furthermore, they are consistent with the study by Gleeson et al [33] who reported higher mucosal salivary IgA levels at 8 and 16 weeks in athletes consuming LcS in addition to a 36% increase in upper respiratory tract infections in athletes consuming the placebo control. These studies are also consistent with human and animal data showing increases in specific IgA antibodies too viral, parasitic and yeast infections associated with LcS or other *Lactobacillus* probiotic intervention [87,88,57,89]. The B cell isotype switch to IgA production is controlled by TGF-β [90] therefore it is possible that via TGF-β induction LcS induces IgA1 and IgA2. Our results for peripheral blood influenza A-specific IgA and IgG and tetanus specific IgG levels during LcS ingestion do not indicate a non-specific stimulatory effect on systemic B cell responses. Although we did not investigate the effect of LcS in relation to vaccination, interestingly a lack of effect of LcS, given at the same dose of 1.3×10^{10} live bacteria per day, was reported for serum anti-influenza antibody titres in a healthy elderly population given the influenza vaccine [91]. However, one reason for this may have been the fact that several of the subjects in the latter study were very elderly. Two other studies, using different *Lactobacillu*s strains and dosing schedules (one in adults [92] and one in aged adults [87]) did, however, show improved antibody responses to influenza vaccination. Future research should therefore be aimed at determining the specificities of the LcS-induced increase in mucosal salivary IgA1 and IgA2 concentration and whether it is associated with TGF-β induction. It would also be interesting to investigate whether mucosal salivary IgA1 and IgA2 responses to vaccination can be enhanced by LcS intervention, and the optimal dose, duration and scheduling of intervention required. Finally, the present study relates to LcS in healthy adults but there are also LcS and other probiotic studies in healthy elderly populations and in relation to immunosenescence [53,93]. Probiotics, including LcS, have different effects on DC, T cell and NK cell functions in younger versus older (> 65 years) subjects. These effects include increased, TNF-α, IL-6, IFN-γ, TGF-β and RANTES production, CD69, CD80 (co-stimulatory signal - ligand for T cell CD28 and CD152) and CD25 (activation and expansion of T cells and NK cells) expression in *in vitro* co-culture experiments most as a function of ageing [93,94], and could potentially have beneficial effects *in vivo* in aged subjects, notwithstanding the age decline in the effective naïve T cell pool [93].

In conclusion, in healthy adults, ingestion of a probiotic drink containing LcS increased expression of the CD69 activation marker on circulating T cells and NK cells and induced a higher mucosal salivary IFN-γ, IgA1 and IgA2 concentration, with evidence of secondary boosting. There was no effect on systemic circulating influenza A specific IgA or IgG and tetanus specific IgG antibody levels with the schedule and dosage used in the current study. Furthermore, LcS induced a decrease in IL-4 in $CD3^+\beta7^+$ integrin cells and a

CD14$^+$ cell anti-inflammatory cytokine profile.

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Figure legends

Figure 1 Mean ±SEM salivary IgA1 concentration (mg/l) in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to10 and LcS resumption from week 10 to 14.

Figure 2 Mean ±SEM salivary IgA2 concentration (mg/ml), in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14.

Figure 3 Mean ±SEM salivary IgA1 secretion rate (μg/minute) in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14.

Figure 4 Mean ±SEM salivary IgA2 secretion rate (μg/minute) in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to10 and LcS resumption from week 10 to 14.

Table 1 Effect of probiotic *Lactobacillus casei* Shirota (LcS) ingestion on T-cell (CD3+) and NK cell (CD16/56+) *ex vivo* stimulated and unstimulated cell surface marker expression in healthy volunteers

Cell surface marker (mean % positively stained cells)	Week 0	SE	Week 4	SE	Week 10
CD3$^+$CD69$^+$ (unstimulated)	0.96	0.46	1.35	0.51	1.96*
CD3$^+$CD69$^+$ (PHA-stimulated)	5.45	2.3	3.08*	1.4	3.17*
CD16/56CD$^+$CD69$^+$(unstimulated)	2.88	1.09	3.19	1.95	4.68
CD16/56CD$^+$CD69$^+$ (PHA-stimulated)	4.87	1.59	4.99	3.92	3.65
CD3$^+$CD16/56$^+$ (unstimulated)	2.63	1.2	4.41*	2.7	9.5**
CD3$^+$CD16/56$^+$ (PHA-stimulated)	4.48	2.65	5.87	2.48	6.99

Data are means ± SEM % positively stained cells for cell surface marker expression of CD3$^+$CD69$^+$ (*ex vivo* unstimulated and PHA-stimulated), CD16/56CD$^+$CD69$^+$ (*ex vivo* unstimulated and PHA-stimulated) and CD3$^+$CD16/56$^+$ (*ex vivo* unstimulated and PHA-stimulated) for 14 healthy volunteers ingesting LcS from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14. Analysed by FACS. * $P<0.05$, ** $P <0.005$, †† $P <0.001$

Table 2 Effect of probiotic *Lactobacillus casei* Shirota (LcS) ingestion on *ex vivo* stimulated and unstimulated intracellular cytokines in healthy volunteers

Cytokine (mean % positively stained cells)	Week 0	SE	Week 4	SE	Week 10	SE	Week 14	SE
$CD3^+\beta7^+IFN\gamma^+$ (PMA, ionomycin-stimulated)	2.52	0.66	1.97	0.6	2.14	0.35	2.09	1.31
$CD3^+\beta7^+IFN\gamma^+$ (unstimulated)	0.24	0.14	0.15	0.04	0.14	0.09	0.13	0.05
$CD3^+\beta7^+IL\text{-}4^+$ (PMA, ionomycin-stimulated)	1.03	0.42	0.89	0.3	1.1	0.5	0.55**	0.4
$CD3^+\beta7^+IL\text{-}4^+$ (unstimulated)	0.46	0.36	0.16	0.03	0.17*	0.09	0.17*	0.07
$CD14^+$ $IL\text{-}12^+$ (LPS-stimulated)	66.91	10.45	41.98††	10.8	43.04**	12.1	42.62††	9.8
$CD14^+$ $IL\text{-}12^+$ (unstimulated)	60.86	12.06	42.49**	14.5	41.97**	10.5	42.02††	9.7
$CD14^+$ $TNF\text{-}\alpha^+$ (LPS-stimulated)	29.27	9.32	13.68***	7.43	13.15†	5.9	21.68*	8.32
$CD14^+$ $TNF\text{-}\alpha^+$ (unstimulated)	0.65	0.29	0.5	0.29	1.69	1.16	1.43	0.64

Data are means ± SEM % positively stained cells for cytokine staining of $CD3^+\beta7^+IFN\gamma^+$(*ex vivo* unstimulated and PMA, ionomycin-stimulated), $CD3^+\beta7^+IL\text{-}4^+$(*ex vivo* unstimulated and PMA, ionomycin-stimulated), $CD14^+$ $IL\text{-}12^+$(*ex vivo* unstimulated and LPS-stimulated) and $CD14^+$ $TNF\text{-}\alpha^+$(*ex vivo* unstimulated and LPS-stimulated) for 14 healthy volunteers ingesting LcS from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14. Analysed by FACS.

* $P<0.05$; ** $P<0.005$; *** $P<0.0005$, † $P<0.01$;

†† $P<0.001$; ††† $P<0.0001$

Table 3 Effect of probiotic *L. casei* Shirota (LcS) ingestion on plasma mean ±SE influenza A-specific IgA (U/ml), IgG (U/ml) and tetanus IgG (IU/ml) in healthy volunteers

Plasma antibodies	Week 0	SE	Week 4	SE	Week 10	SE	Week 14	SE
Influenza A IgA (n=14)	15.56	10.84	15.45	9.9	17.09	11.9	16.26	10.1
Influenza A IgG (n=7)	2.59	0.28	2.57	0.36	2.58	0.34	2.68	0.4
Tetanus IgG (n=7)	4.0	0.24	4.10	0.18	4.24	0.14	4.27	0.19

Data are means ± SEM for plasma influenza A-specific IgA (U/ml), influenza A-specific IgG (U/ml) and tetanus IgG (IU/ml) for healthy volunteers ingesting LcS from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14. Analysed by ELISA.

Figure 1 Mean ±SEM salivary IgA1 concentration (mg/l) in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14.
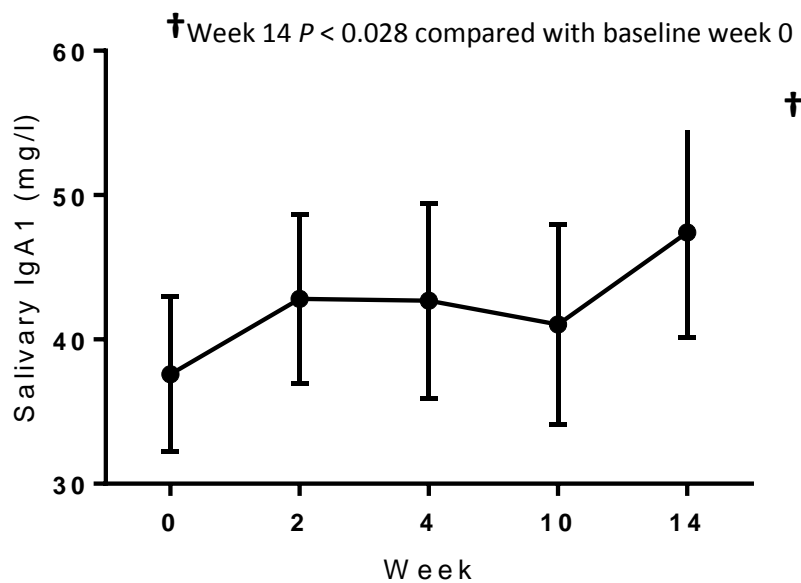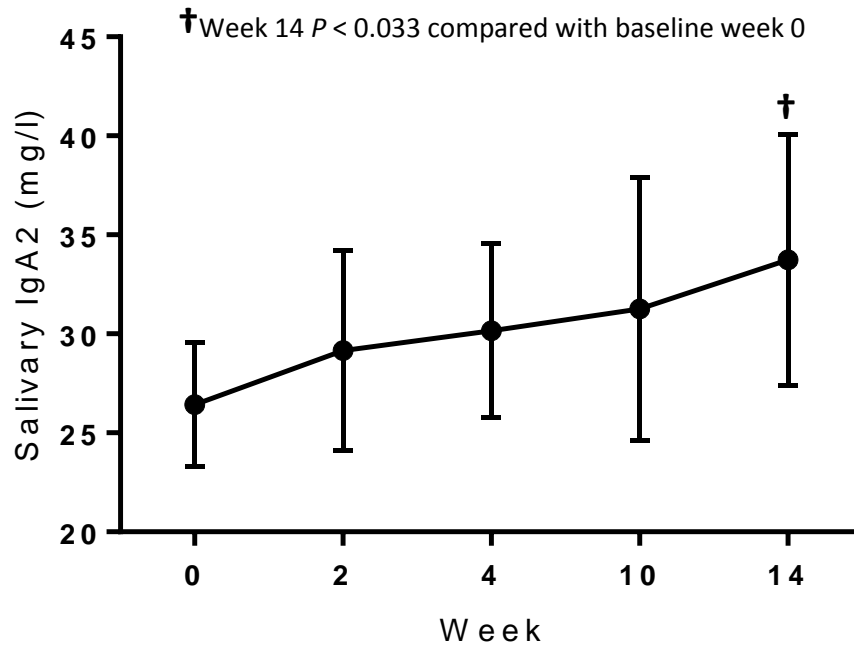

† Week 14 $P < 0.028$ compared with baseline week 0

Figure 2 Mean ±SEM salivary IgA2 concentration (mg/ml), in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14.

Figure 3 Mean ±SEM salivary IgA1 secretion rate (µg/minute) in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14.
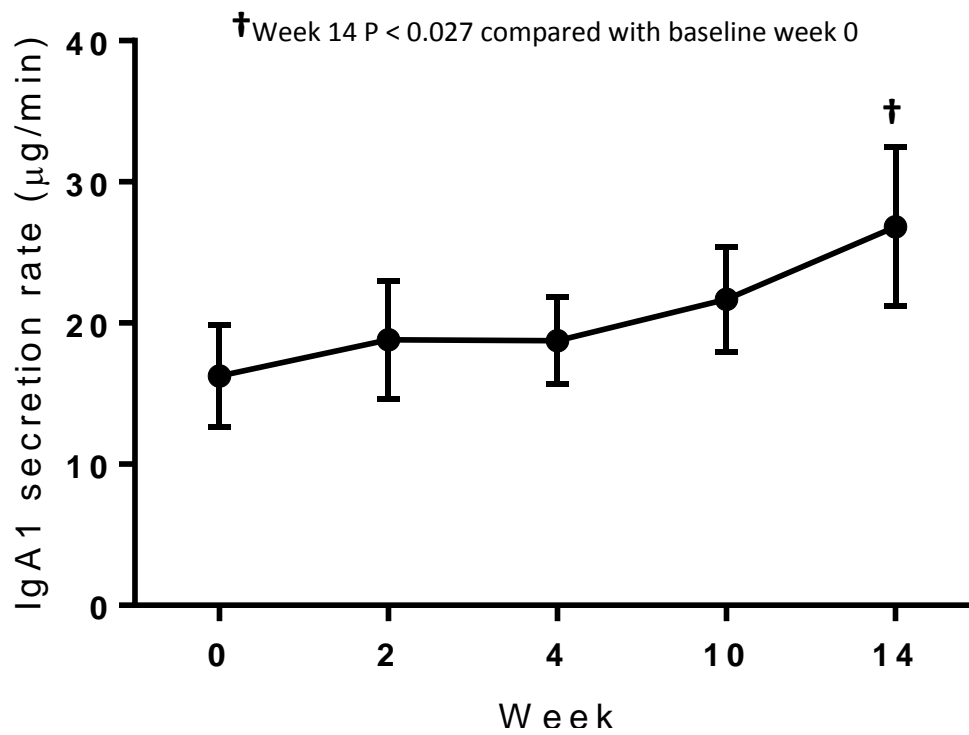
Figure 4 Mean ±SEM salivary IgA2 secretion rate (μg/minute) in healthy volunteers (n=10) ingesting the probiotic *Lactobacillus casei* Shirota (LcS) from week 0 to 4, discontinuation of LcS ingestion from week 4 to 10 and LcS resumption from week 10 to 14.