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# β2-1 Fructans have a bifidogenic effect in healthy middle-aged human subjects but do not alter immune responses examined in the absence of an *in vivo* immune challenge: results from a randomised controlled trial

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## Abstract

β2-1 fructans are considered to be prebiotics. Current literature indicates that β2-1 fructans may modulate some aspects of immune function, improve the host's ability to respond to certain intestinal infections, and modify some inflammatory outcomes in human subjects. However, there is a need to find out more about the modulation of immune markers by β2-1 fructans in humans. Healthy human subjects aged 45–65 years were randomly allocated to β2-1 fructans (Orafti® Synergy1; 8 g/d; *n* 22) or the digestible carbohydrate maltodextrin as placebo (*n* 21) for 4 weeks. Blood, saliva and faecal samples were collected at study entry and after 4 weeks. Immune parameters were measured using the blood and saliva samples and bifidobacteria were measured in the faecal samples. Faecal bifidobacteria numbers increased in the Orafti® Synergy1 group ($P<0{\cdot}001$) and were different at 4 weeks from numbers in the placebo group ($P=0{\cdot}001$). There was no significant effect of Orafti® Synergy1 on any of the immune parameters measured (blood immune cell subsets, total serum Ig, salivary IgA, neutrophil and monocyte phagocytosis of *Escherichia coli* and respiratory burst in response to *E. coli* or phorbol ester, natural killer cell activity, T cell activation and proliferation, production of six cytokines by T cells). It is concluded that, compared with maltodextrin, Orafti® Synergy1 has a bifidogenic effect in healthy middle-aged human subjects but does not alter immune responses examined in the absence of an *in vivo* immune challenge.

**Key words: Prebiotics: β2-1 Fructans: Immune function: Bifidobacteria**

The prebiotic concept has been defined as 'the selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host'[1]. β2-1 Fructans are considered to be prebiotics. The naturally occurring parent β2-1 fructan molecule is inulin. Inulin can vary in chain length, and it can also be digested to lower-molecular-weight (shorter-chain-length) molecules. Low-molecular-weight β2-1 fructans can also be synthesised chemically. Here we refer to both inulin-derived and chemically synthesised short-chain-length β2-1 fructans as shorter-chain oligofructose. Orafti® Synergy1 (hereafter referred to as Synergy1) contains a 50:50 (w/w) mixture of long-chain inulin and shorter-chain oligofructose. β2-1 Fructans have been reported to modulate the intestinal microbiota, specifically increasing the numbers of bifidobacteria[2–7], lactobacilli[5] and *Faecalibacterium prausnitzii*[8,9]. It is thought that these types of bacteria influence the host immune system, improving its function. Studies performed to date have indicated that β2-1 fructan supplementation may modulate aspects of immune function in children[10–13], young adults[14], elderly adults[2,15–19], and adults with colon cancer[20,21], active ulcerative colitis[22] or in an intensive care unit[23]. However, many of these studies have combined β2-1 fructans with other nutrients[11,12,14,17–23], and they have evaluated different immune parameters. Therefore, it is difficult to reach a firm conclusion about the impact of β2-1 fructans on immune function in humans[24]. There are no studies

**Abbreviations:** CFSE, carboxyfluorescein succinimidyl ester; ConA, concanavalin A; FCS, fetal calf serum; FIFC, fluorescein isothiocyanate; MFI, median fluorescence intensity; PBMC, peripheral blood mononuclear cells; RPMI, Roswell Park Memorial Institute; sIgA, secretory IgA.

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of the mixture of inulin and shorter-chain oligofructose, as found in Synergy1, and no studies evaluating the effect of β2-1 fructans on immune parameters in healthy middle-aged human subjects. Therefore, the aim of the present study was to evaluate the effect of Synergy1 on faecal bifidobacteria and immune parameters in this target group using a double-blind randomised controlled trial design with the digestible carbohydrate maltodextrin as placebo. It was anticipated that Synergy1 would increase faecal bifidobacteria numbers and would enhance some of the immune parameters measured; markers of both innate and acquired immunity were measured.

## Subjects, materials and methods

### Subjects

Subjects ($n$ 49) were recruited via posters, word of mouth, email and newspaper/magazine advertisements. The inclusion criteria were as follows: age between 45 and 65 years; BMI between 20 and 32 kg/m$^2$; not consuming prebiotic or probiotic supplements, drinks or foods; in general good health; no antibiotic use in the 2 months before entering the study or during the study; and not having been vaccinated with the current season's (2008/2009) influenza vaccination; this is because the subjects involved in the study subsequently received this vaccination (data not reported here). Both men and women were recruited. The exclusion criteria were as follows: age $<45$ or $>65$ years; BMI $<20$ or $>32$ kg/m$^2$; being diabetic (type 1 or type 2); displaying manifestations of allergy (asthma, hay fever or dermatitis) or being treated for these; being egg allergic; use of any prescribed medication (unless deemed acceptable by the principal investigator); suffering from any infectious illness or chronic gastrointestinal problem (e.g. irritable bowel syndrome, inflammatory bowel disease, cancer); recent blood donation; participation in another clinical trial; use of prebiotic or probiotic supplement foods or drinks; consuming vitamin, fish oil, evening primrose oil or mineral supplements; previously vaccinated with the 2008/2009 vaccination or a previous influenza vaccination which contained any of the strains included in the 2008/2009 vaccine. The study was registered as 'Prebiotics and Immune Function in Middle Aged Humans' on www.clinicaltrials.gov (study identifier: NCT00898599). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Southampton and South West Hampshire Local Research Ethics Committee (09/H0504/2). Written informed consent was obtained from all subjects. Clinical governance was provided by the Southampton University Hospitals NHS Trust R&D, and the study was performed according to good clinical practice.

### Study design and intervention

The study was a randomised, double-blind, placebo-controlled trial, with an 8-week intervention period; only data from the first 4 weeks are reported here. Randomisation to the β2-1 fructan ($n$ 25) or placebo (maltodextrin; $n$ 24) group was done by random selection of a sealed envelope containing a treatment code. Unblinding did not occur until after completion of all statistical analyses. Following randomisation, subjects underwent a 2-week run-in period, during which they limited their consumption of prebiotic- and probiotic-containing foods, and these restrictions continued throughout the study. Upon completion of the run-in period, subjects began the 8-week supplementation period; the start of this period is referred to as week 0. Synergy1 and maltodextrin were provided as powders within coded, sealed paper sachets (4 g/sachet) by Beneo-Orafti, and were identical in appearance and packaging, except for the labelling (A or B). Subjects were asked to consume two sachets per d (one in the morning and one in the evening; total intake 8 g/d) by stirring the contents into a glass of water. Subjects were given enough sachets to cover the period of the study; they were asked to return unused and used sachets for assessment of compliance. Subjects attended a clinic at the Wellcome Trust Clinical Research Facility, Southampton General Hospital at weeks 0 and 4 at which blood, saliva and faecal samples were collected; at week 4, the subjects received the 2008/2009 seasonal influenza vaccination but data beyond week 4 are not reported here.

### Assessment of subjects' self-reported gastrointestinal sensations

At the clinic visit at week 4, subjects were verbally asked to report any gastrointestinal sensations or health problems they had encountered in the previous 4 weeks. Specifically, they were asked the question 'Have you noticed any changes in your bowel habits, for example, changes in bowel movements, bloating, gas, unusual pain etc.?'. If they answered 'yes' but did not elaborate, they were asked a second question: 'what changes have you noticed?'.

### Enumeration of faecal total bacteria and bifidobacteria

Frozen (−80°C) faecal samples collected at the two clinic visits were thawed and then diluted (1:10) in PBS and homogenised in a stomacher for 120 s. The samples were centrifuged at 1500 rpm for 2 min, to separate the liquid and solid matter. An aliquot of the liquid was used to enumerate total bacteria and bifidobacteria. For the total bacteria, the liquid (375 μl) was fixed for 4 h at 4°C with 1·125 ml of 4 % (w/v) paraformaldehyde; the fixed samples were centrifuged at 13 000 rpm for 5 min, and washed twice with PBS. The washed cells were resuspended in a mixture of 150 μl PBS and 150 μl ethanol, and stored at −20°C until further processing. The samples were then diluted in a suitable volume of sterile PBS/10 % SDS in order to obtain an appropriate number of cells per microscopic field of view (a dilution of 1:400 or 1:1000). Then, 20 μl of this sample were added to each well of a six-well slide (Teflon- and poly-L-lysine-coated, six-well, 10 mm-diameter well slides; Tekdon, Inc.). The samples were dried for approximately 15 min in a drying chamber at 46–50°C. They were then dehydrated using an alcohol washing series

(50, 80 and 96 % (v/v) ethanol) for 3 min with each solution. Slides were dried for 2 min at 46–50°C to evaporate the excess ethanol. The nucleic acid stain 4′,6-diamidino-2-phenylindole (50 ng/μl) was added to PBS at a dilution of 1:11, and 50 μl of this mixture were applied to each well. The slides were incubated at room temperature in the dark for 15 min and were then washed in 50 ml of washing buffer (40 ml double-distilled water, 1 ml of 1 M-Tris–HCl (pH 8·0) and 9 ml of 5 M-NaCl) for 15 min at 50°C. They were then dipped into ice-cold distilled water for 10 s, and dried with compressed air. Then, 5 μl of antifade reagent were added to each well, and a coverslip applied to the slide. The slides were stored in the dark at 4°C until analysis. The slides were counted using an epifluorescence Brunel microscope. Filters for the 4′,6-diamidino-2-phenylindole stain (excitation at 550 nm and emission at 461 nm) and the Cy3 dye (excitation at 550 nm and emission at 564 nm) were used. For each sample, fifteen fields of view were counted. Total bacteria counts were calculated taking into consideration the dilution factors used.

For enumeration of bifidobacteria, samples of the faecal liquid (375 μl) were centrifuged at 13 000 rpm for 5 min. The cell pellet was resuspended in a mixture of 150 μl PBS and 150 μl ethanol, and stored at −20°C until further processing. Processing was as described above for total bacteria up to the completion of the ethanol washes of the slides. Then, a synthetic oligonucleotide probe targeting specific regions of the 16S rRNA labelled with the fluorescent dye Cy3 was used for the enumeration of the *Bifidobacterium* genus (probe: Bif 164, target sequence: CATCCGGCATTACCACCC). 5 μl of the probe (50 ng/μl) and 50 μl of the hybridisation buffer (799 μl double-distilled water, 20 μl of 1 M-Tris–HCl (pH 8·0), 180 μl of 5 M-NaCl and 1 μl SDS) were added to each well and allowed to hybridise for 4 h in a microarray hybridisation incubator at 50°C. Following hybridisation, slides were washed in 50 ml of washing buffer (40 ml double-distilled water, 1 ml of 1 M-Tris–HCl (pH 8·0), 9 ml of 5 M-NaCl and 20 μl of 4′,6-diamidino-2-phenylindole (50 ng/μl)) for 15 min at 50°C. Further processing was as described above for total bacteria.

### Measurement of salivary IgA concentration

Subjects chewed on a dental roll for approximately 30 s. Dental rolls were kept on ice until processing and were then transferred to the barrel of a 5 ml syringe. Saliva was collected by pressure, centrifuged (2000 rpm, 10 min) to remove debris, and stored at −80°C until analysis. Secretory IgA (sIgA) concentrations were measured by ELISA (Demeditec). The limit of detection was 1 μg/ml. Total salivary protein concentration was measured using the Bradford assay; salivary sIgA concentration is expressed both unadjusted and adjusted for total protein.

### Assessment of blood immune cell phenotypes

Fresh heparinised blood (100 μl) was incubated for 30 min in the dark at 4°C with the following fluorescently conjugated antibodies: anti-CD3(fluorescein isothiocyanate (FIFC))/anti-CD4(RPE) to identify helper T cells; anti-CD3(FITC)/anti-CD8(RPE) to identify cytotoxic T cells; anti-CD3(FITC)/anti-CD16(RPE) to identify natural killer cells; anti-CD3(FITC)/anti-CD19(RPE) to identify B cells; anti-CD14 (FITC) to identify monocytes; anti-CD4(FITC)/anti-CD25(PECy5)/anti-CD127(PE) to identify regulatory T cells. All antibodies were purchased from AbD Serotec. Following incubation, erythrocytes were lysed (cell lysis buffer; BD Biosciences) and incubated for a further 10 min in the dark at 4°C. Samples were then centrifuged (1000 rpm, 7 min), and the cells were washed (cell wash solution; BD Biosciences) at 1000 rpm for 7 min. Cells were fixed in 200 μl cell fix solution (BD Biosciences), and kept at 4°C until analysis by flow cytometry within 48 h. Appropriate isotype controls were always included. Flow cytometry was performed using a Becton Dickinson FACSCalibur fluorescence-activated cell sorter; in all cases, 10 000 events were collected. Results were analysed using CellQuest software (BD Biosciences).

### Measurement of leucocyte phagocytosis and oxidative burst

Phagocytosis of *Escherichia coli* by neutrophils and monocytes and respiratory burst of neutrophils and monocytes in response to *E. coli* or phorbol myristyl acetate were measured in heparinised whole blood using Phagotest and Phagoburst kits, respectively (both from Orpegen Pharma). In both cases, the manufacturer's instructions were followed. Samples were analysed by flow cytometry on a Becton Dickinson FACSCalibur fluorescence-activated cell sorter and data were collected for 10 000 cells. Results were analysed using CellQuest software. The percentage of neutrophils and monocytes performing phagocytosis, the number of ingested bacteria per cell (mean fluorescence intensity), the percentage neutrophils and monocytes producing reactive oxygen metabolites, and the extent of production (mean fluorescence intensity) were all determined.

### Serum and peripheral blood mononuclear cell preparation

Blood (5 ml) was collected into serum tubes and kept at room temperature until processing. The samples were centrifuged (2000 rpm, 10 min, 4°C), and serum was stored at −80°C until analysis. Blood (35 ml) was collected into heparin tubes and kept at room temperature until processing. A 2 ml sample of the whole blood was removed and used for immune cell phenotyping, and for the Phagotest and Phagoburst assays (see above). The remaining whole blood was layered onto an equal volume of Histopaque, density 1·077 g/ml (Sigma), and centrifuged (2000 rpm, 15 min, room temperature). Peripheral blood mononuclear cells (PBMC) were removed from the interface and washed with Roswell Park Memorial Institute (RPMI)-1640 medium containing 10μg penicillin/ml, 100 μg streptomycin/ml and 2 mM-glutamine (all from Sigma). The PBMC were then resuspended in RPMI-1640 medium plus 2 % fetal calf serum (FCS; PAA) and washed twice (1200 rpm, 10 min, room temperature). The PBMC were then resuspended in 1 ml RPMI plus 2 % FCS, counted using Trypan Blue staining on a haemocytometer and adjusted to an appropriate cell

concentration for cryopreservation. An equal volume of freezing medium (FCS plus 15% dimethyl sulphoxide (Fischer) at 4°C) was added dropwise to the cell suspension on ice to make a final concentration of between $10 \times 10^6$ and $25 \times 10^6$ cells/ml. Cells were then stored at −196°C in a liquid $N_2$ tank until use in further experiments. Cryopreserved cells were thawed rapidly in a water-bath at 37°C. The cells were then placed on ice, and ice-cold RPMI was added dropwise for 1 min and the volume then made up to 10 ml. The cells were centrifuged (1200 rpm, 7 min, room temperature), and then resuspended at a density of $1 \times 10^6$/ml in RPMI-1640 medium containing 10% FCS.

### Measurement of total serum Ig

Serum concentrations of IgA, IgM and IgG were measured by ELISA (Zeptometrix for IgA and IgM; Immunodiagnostik for IgG). In all cases, the manufacturers' instructions were followed. Limits of detection were as follows: IgA and IgM 7·8 ng/ml; IgG 0·32 μg/ml.

### Measurement of natural killer cell activity

Natural killer cell activity was determined as killing of the K562 (target) cell line by PBMC. PBMC were used as prepared above and were also pre-incubated with IL-2 (200 U/ml) for 21 h at 37°C in a 5% $CO_2$ atmosphere. Target cells ($5 \times 10^6$/ml) were incubated with 1·5 μl carboxyfluorescein succinimidyl ester (CFSE; 5 mg/ml final concentration) for 45 min at 37°C in a 5% $CO_2$ atmosphere. They were then washed and resuspended in RPMI-1640 medium supplemented with 2 mM-glutamine, antibiotics (10 μg penicillin/ml and 100 μg streptomycin/ml) and 10% FCS. Target cells ($1 \times 10^4$ in a volume of 100 μl) were then incubated with PBMC at PBMC:target cell ratios of 100:1, 50:1, 25:1 and 12·5:1 and in a total volume of 300 μl. Incubation was for 2·5 h at 37°C in a 5% $CO_2$ atmosphere. Cultures without PBMC were included to calculate spontaneous K562 death. After incubation, the cells were collected and transferred to flow cytometry tubes; propidium iodide (final concentration 1 mg/ml) was added to each tube. Cells were then analysed by flow cytometry using a Becton Dickinson FACSCalibur fluorescence-activated cell sorter; in all cases, 10 000 events were collected. Results were analysed using CellQuest software (BD Biosciences); dead K562 cells were identified as propidium iodide$^+$ CFSE$^+$. Specific cell lysis was calculated as (percentage of total target cell death − percentage of spontaneous target cell death).

### Measurement of T cell activation by CD69 expression

Activation of $CD4^+$ T cells was determined by the appearance of CD69 on the cell surface following stimulation with the T cell mitogen concanavalin A (ConA). PBMC were resuspended at $1 \times 10^6$ cells/ml in RPMI-1640 medium plus 10% FCS. They were cultured in ninety-six-well plates for 24 h at 37°C in a 5% $CO_2$ atmosphere. Total culture volume was 200 μl ($2 \times 10^5$ cells/well), consisting of RPMI-1640 medium supplemented with 2 mM-glutamine, antibiotics (10 μg penicillin/ml and 100 μg streptomycin/ml), 10% FCS and ConA (5 μg/ml); unstimulated cultures were also performed. After 24 h of culture, plates were centrifuged (1000 rpm, 10 min), and the cells collected. The cells were incubated for 30 min in the dark at 4°C with 10 μl anti-CD3(FITC)/anti-CD4(RPE) + 5 μl anti-CD69(PECy5) or anti-CD3(FITC)/anti-CD4(RPE) + 10 μl IgG2a(PECy5) as an isotype control for anti-CD69. The samples were then centrifuged (1000 rpm, 7 min), and the cells washed (cell wash solution; BD Biosciences) at 1000 rpm for 7 min. The cells were fixed in 200 μl cell fix solution (BD Biosciences), and kept at 4°C until analysis by flow cytometry within 48 h. Appropriate isotype controls were always included. Flow cytometry was performed using a Becton Dickinson FACSCalibur fluorescence-activated cell sorter; in all cases, 10 000 events were collected. Results were analysed using CellQuest software (BD Biosciences). The percentage of cells that had undergone activation (i.e. $CD3^+CD4^+CD69^+$) as well as the median fluorescence intensity (MFI) of CD69 expression on these cells were determined.

### Measurement of lymphocyte proliferation by carboxyfluorescein succinimidyl ester dilution

Proliferation of lymphocytes was determined by the dilution of CFSE following ConA stimulation. PBMC were resuspended at $1 \times 10^6$ cells/ml in RPMI medium plus 10% FCS. CFSE stock solution (Molecular Probes) was added to give a final concentration of 10 μM. Cells were then incubated for 10 min at 37°C in a 5% $CO_2$ atmosphere. Staining was quenched by adding five volumes of ice-cold medium and incubating the cells on ice for 5 min. The cells were then washed and resuspended at $1 \times 10^6$/ml in RPMI-1640 medium plus 10% FCS. They were cultured in ninety-six-well plates for 168 h at 37°C in a 5% $CO_2$ atmosphere. Total culture volume was 200 μl ($2 \times 10^5$ cells/well), consisting of RPMI-1640 medium supplemented with 2 mM-glutamine, antibiotics (10 μg penicillin/ml and 100 μg streptomycin/ml), 10% FCS and ConA (5 μg/ml); unstimulated cultures were also performed. After 168 h, the cells were collected, transferred to flow cytometry tubes and analysed using a Becton Dickinson FACSCalibur fluorescence-activated cell sorter. Results were analysed using FlowJo software (Tree Star, Inc.). The percentage of cells that had undergone proliferation as well as CFSE MFI were determined.

### Measurement of cytokine production by peripheral blood mononuclear cells

PBMC were cultured as described above for the measurement of T cell activation. After 24 h of culture, the culture plates were centrifuged (1000 rpm, 10 min), and the supernatants removed and stored at −20°C until further analysis. Concentrations of IL-2, IL-4, IL-6, IL-10, TNF-α and interferon γ were measured using the human Th1/Th2 cytokine kit II (cytometric bead array; BD Biosciences). Culture supernatants (25 μl) were added to tubes containing 25 μl of the cytokine capture beads and 25 μl of detection reagent, and incubated for 3 h. After washing, data were collected on a Becton

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Dickinson FACSCalibur fluorescence-activated cell sorter and captured using CellQuest software. Analysis was performed with FCAP Array software (BD Biosciences). Cytokine standards from a concentration of 0 to 20 000 pg/ml were run in parallel. Limits of detection were as follows: IL-2, 2·6 pg/ml; IL-4, 2·6 pg/ml; IL-6, 3·0 pg/ml; IL-10, 2·8 pg/ml; TNF-α, 2·8 pg/ml; interferon γ, 7·1 pg/ml.

### Statistical analysis

Comparisons between the groups were performed using the independent samples *t* test, Mann–Whitney test or $\chi^2$ test depending upon the nature of the data. Comparisons between time points within a group were made using the paired *t* test or Wilcoxon signed-rank test depending upon the nature of the data. Spearman's correlation coefficients were calculated to determine the association between BMI and various outcomes. All analyses were performed using SPSS version 17.0 (SPSS, Inc.), and in all cases, a value of $P<0·05$ was taken to indicate statistical significance.

## Results

### Subject characteristics and compliance

Of the forty-nine subjects recruited and randomised (*n* 24 in the maltodextrin group; *n* 25 in the prebiotic group), five withdrew before the intervention started. Later, one subject withdrew once the intervention started; this subject was in the prebiotic group and reported inability to tolerate unpleasant gastrointestinal sensations (bloating, unease and feeling urgency to empty bowels). Therefore, forty-three subjects completed the study (*n* 21 in the maltodextrin group; *n* 22 in the prebiotic group). The characteristics of these subjects did not differ between the groups (Table 1), although there was a trend towards a different balance of sexes between the two groups ($P=0·066$). The proportion of subjects with a BMI of $<25$ kg/m$^2$ did not differ between the two groups (52% in both groups). Compliance, assessed by returned unused sachets, was good in both groups, and ranged from 87 to 101% (median 100%) in the maltodextrin group and 74 to 100% (median 100%) in the prebiotic group.

### Self-reported gastrointestinal sensations

Gastrointestinal sensations reported among subjects who completed the study are shown in Table 2. More subjects in the prebiotic group reported increased flatulence and increased regularity of bowel movements.

**Table 1.** Subject characteristics according to the study group*

| | Placebo group | Prebiotic group |
|---|---|---|
| *n* | 21 | 22 |
| Age (years) | | |
| Mean | 56 | 54 |
| Range | 45–63 | 45–62 |
| Male:female | 8:13 | 3:19 |
| BMI (kg/m$^2$) | | |
| Mean | 25·0 | 25·7 |
| Range | 17·7–33·8 | 19·4–33·3 |

*Data are shown for subjects who completed the study.

**Table 2.** Self-reported gastrointestinal sensations in subjects receiving placebo or prebiotic for 4 weeks

(Number of subjects reporting each sensation)

| Sensation | Placebo group (*n*) | Prebiotic group (*n*) |
|---|---|---|
| Increased bloating | 1 | 2 |
| Decreased bloating | 0 | 1 |
| Increased flatulence | 1 | 12** |
| Increased regularity of bowel movements | 2 | 9* |
| Increased constipation | 2 | 2 |
| Decreased constipation | 0 | 1 |
| Looser stools | 0 | 3 |

*Values were significantly different from the placebo group ($\chi^2$ test, $P=0·034$).
**Values were significantly different from the placebo group ($\chi^2$ test, $P=0·001$).

### Total bacteria and bifidobacteria

Fig. 1 shows faecal total bacteria and bifidobacteria numbers in each group at study entry (week 0) and after 4 weeks. At week 0, there were no differences between the groups regarding the number of bifidobacteria (placebo: $1·58\times10^9$/g faeces; prebiotic: $1·33\times10^9$/g faeces), the total number of bacteria (placebo: $0·72\times10^{11}$/g faeces; prebiotic: $1·01\times10^{11}$/g faeces) or bifidobacteria expressed as a percentage of total bacteria (average of 1·25% in both groups). There was no change in total bacteria or bifidobacteria in the placebo group or in total bacteria in the prebiotic group (Fig. 1). However, in the prebiotic group, the number of bifidobacteria was significantly higher ($P<0·001$) at week 4 ($2·82\times10^9$/g faeces) than at week 0, and was significantly higher ($P=0·001$) than in the placebo group at week 4 ($1·14\times10^9$/g faeces). The change in bifidobacteria number in the prebiotic group between week 0 and week 4 (median increase $1·26\times10^9$/g faeces) was significantly different ($P=0·001$) from the change in the placebo group (median increase $3·37\times10^7$/g faeces). Bifidobacteria as a percentage of total bacteria were significantly higher ($P=0·001$) in the prebiotic group at week 4 (2·64%) compared with week 0. There was a significantly greater ($P=0·002$) change in bifidobacteria as a percentage of total bacteria in the prebiotic group (median increase 0·84%) compared with the placebo group (median increase 0·05%).

### Blood immune cell phenotypes

Immune cell phenotypes did not differ between the groups at week 0 (Table 3). In the placebo group, the percentage of $CD3^-CD16^+$ cells decreased ($P=0·023$) and $CD14^+$ cells expressed as a percentage of monocytes increased (63·9–77·9%, $P=0·020$), but this did not occur in the prebiotic group. The percentages of $CD3^+CD8^+$ and $CD8^+$ cells were higher in the prebiotic group at week 4 compared with the placebo group ($P=0·032$ and 0·023, respectively). When comparing the changes over time between the groups, there were no significant differences. Therefore, it must be concluded that the prebiotic supplement had no effect upon immune cell phenotypes in the blood.

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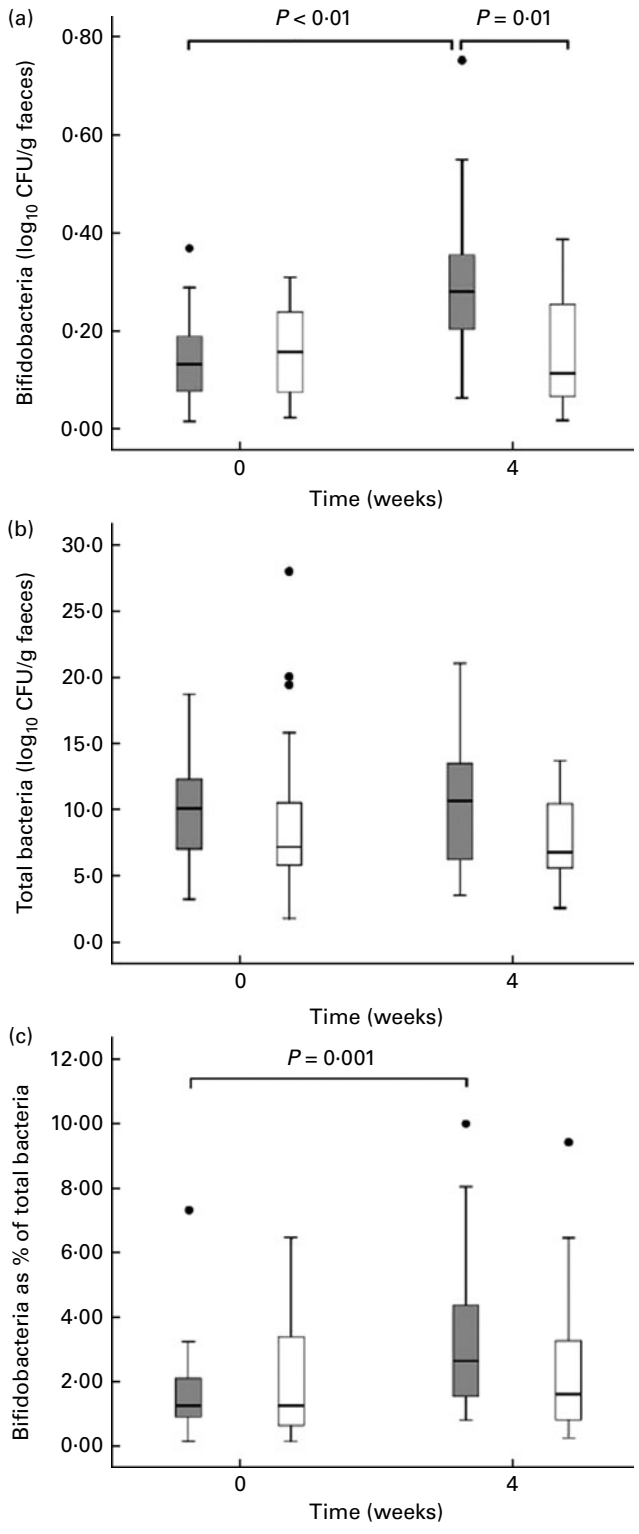


**Fig. 1.** Faecal bacteria in subjects receiving placebo or prebiotic for 4 weeks. (a) Bifidobacteria; (b) total bacteria; (c) bifidobacteria as a percentage of total bacteria. Box plots show median, first and third quartiles, minimum and maximum values, and outliers. □, Placebo group; ■, prebiotic group. Significant differences determined by the Mann–Whitney test and Wilcoxon signed-rank test. CFU, colony-forming units.

### *Markers of innate immunity: phagocytosis and oxidative burst by neutrophils and monocytes and natural killer cell activity*

Data for phagocytosis of *E. coli* by neutrophils and monocytes, respiratory burst by neutrophils and monocytes in response to *E. coli* or phorbol myristyl acetate, and natural killer cell activity of PBMC towards K562 cells with and without prior incubation with IL-2 are shown in Table 4. Pre-incubation with IL-2 enhanced natural killer cell activity. Although there were changes over time in some of these measures, these changes were similar in both groups, and there were no differences between the two groups at week 0 or week 4, and there were no differences in the change over time between the groups. Therefore, it must be concluded that the prebiotic supplement had no effect upon these measures of innate immunity.

### *Markers of humoral immunity: total serum Ig and salivary IgA concentrations*

Serum concentrations of IgA, IgG and IgM and salivary sIgA concentration are shown in Table 5. Although there were changes over time in some of these measures, these changes were similar in both groups, and there were no differences between the two groups at week 0 or week 4, and there were no differences in the change over time between the groups. Therefore, it must be concluded that the prebiotic supplement had no effect upon these measures of humoral immunity.

### *Markers of acquired immunity: T cell activation, proliferation and cytokine production*

T cell responses were determined as the expression of the activation marker CD69 on the surface of $CD3^+CD4^+$ cells, dilution of the intracellular dye CFSE in response to a mitogenic signal that promotes proliferation, and production of Th1- and Th2-type cytokines. The T cell mitogen ConA was used to elicit these responses. ConA resulted in a marked increase in CD69-positive $CD3^+CD4^+$ cells and a marked increase in the level of CD69 expression on those cells (i.e. MFI), a marked increase in the percentage of proliferating T cells, and a marked reduction in CFSE MFI indicative of dilution of the dye, and a marked increase in the production of all six cytokines assessed (Table 6). Increases were approximately 5-fold for the percentage of $CD69^+$ cells, approximately 10-fold for the percentage of proliferating cells, and approximately 5- to 250-fold for cytokine production depending upon the cytokine. There were no differences between the two groups at week 0 or week 4 in any of these measures of T cell function, and there were no differences in the change over time between the groups. Therefore, it must be concluded that the prebiotic supplement had no effect upon these measures of T cell function.

### *Associations between BMI and the reported outcomes and the response to β2-1 fructans*

Using data from both groups combined, there was a trend towards an inverse relationship between BMI and bifidobacteria as a

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**Table 3.** Blood immune cell phenotypes in subjects receiving placebo or prebiotic for 4 weeks
(Mean values with their standard errors)

| | Placebo group | | | | Prebiotic group | | | |
|---|---|---|---|---|---|---|---|---|
| | Week 0 | | Week 4 | | Week 0 | | Week 4 | |
| Cell phenotype | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| $CD3^+CD4^+$(% of lymphocytes) | 50·9 | 8·4 | 50·5 | 6·9 | 50·9 | 6·8 | 50·1 | 8·4 |
| $CD4^+$(% of lymphocytes) | 51·2 | 8·3 | 50·9 | 7·1 | 51·2 | 6·9 | 50·3 | 8·3 |
| $CD3^+CD8^+$(% of lymphocytes) | 17·9 | 7·5 | 17·3 | 7·1 | 22·8 | 9·6 | 22·9† | 8·9 |
| $CD8^+$(% of lymphocytes) | 21·3 | 8·2 | 21·0 | 7·4 | 26·3 | 8·8 | 26·7† | 8·0 |
| $CD3^-CD16^+$(% of lymphocytes) | 10·7 | 4·3 | 9·4* | 3·7 | 9·6 | 5·6 | 8·2 | 5·5 |
| $CD3^-CD19^+$(% of lymphocytes) | 10·4 | 3·5 | 10·8 | 3·7 | 9·4 | 3·7 | 10·0 | 3·9 |
| $CD14^+$(% of monocytes) | 63·9 | 24·8 | 77·9* | 13·8 | 74·7 | 15·2 | 76·9 | 14·3 |
| $CD14^+$(% of leucocytes) | 3·2 | 0·9 | 3·6 | 1·5 | 3·2 | 1·0 | 3·2 | 0·6 |
| $CD127^{lo}$ (% of $CD4^+CD25^+$cells) | 69·5 | 7·4 | 73·4 | 8·3 | 73·8 | 6·8 | 73·1 | 8·6 |
| $CD4^+CD25^+CD127^{lo}$ (% of leucocytes) | 1·1 | 0·4 | 1·2 | 0·6 | 1·0 | 0·4 | 1·2 | 0·5 |
| $CD4^+$:$CD8^+$ | 2·8 | 1·2 | 2·8 | 1·1 | 2·2 | 0·9 | 2·1† | 0·9 |

$CD127^{lo}$, $CD127^+$ cells with low fluorescence intensity.
* Mean values were significantly different significantly different from week 0 (paired *t* test) ($P<0·05$).
† Mean values were significantly different from the placebo group at the same time point (independent samples *t* test) ($P<0·05$).

proportion of total faecal bacteria at week 0 ($r$ −0·281, $P=0·092$), but there was no association with the absolute number of bifidobacteria ($r$ −0·233, $P=0·166$). Associations between BMI and the various immune outcomes were explored at week 0. A total of fifty-six associations were explored. Most of these were far from significant, but there were significant associations with the proportion of $CD3^+CD4^+CD69^+$ cells in the bloodstream ($r$ 0·460, $P=0·003$) and CFSE MFI for unstimulated cells ($r$ 0·329, $P=0·033$). IL-6 production by unstimulated and ConA-stimulated T cells tended to be positively associated with BMI ($r$ 0·289, $P=0·060$ and $r$ 0·262, $P=0·090$, respectively).

The change in the various outcomes (i.e. week 4 minus week 0) in the prebiotic group was not different between the subjects with BMI $<25\,kg/m^2$ and those with BMI $>25\,kg/m^2$.

## Discussion

There have been several human studies showing that β2-1 fructan-type prebiotics may have an impact on immune function[2,10–23]. However, these studies have frequently combined the prebiotic with other nutrients[11,12,14,17–23], or have studied infants[10–12] or patients[20–23]. It is necessary to identify the functional effects of β2-1 fructans on immune function in the absence of co-interventions, and to identify whether effects occur in healthy middle-aged subjects since these are a target consumer population. The present study investigated the effect of Synergy1, a mixture of shorter-chain oligofructose and inulin, at 8 g/d for 4 weeks on faecal bifidobacteria and measures of innate, humoral and T cell-mediated immunity in healthy middle-aged subjects; a randomised controlled parallel study design was used with the digestible carbohydrate maltodextrin as placebo. Compliance to both prebiotic and maltodextrin was good. Subjects in the prebiotic group reported altered gastrointestinal sensations, especially increased gas and regularity of bowel movements. These are suggestive of a change in gut microbiota. While increased flatulence may be unpleasant, increased regularity of bowel movements may be a desirable outcome both personally and clinically[25,26], and has recently been endorsed as a beneficial physiological effect by the European Food Standards Authority[27]. Faecal bifidobacteria numbers increased with Synergy1 consumption for 4 weeks. Other studies have previously reported the bifidogenic property of inulin[3–6,28], shorter-chain oligofructose[2,7,29] and Synergy1[30]. In the present study, there was an approximate doubling of faecal bifidobacteria numbers in the Synergy1 group. A similar size of effect has been observed in some studies using inulin[3,4]. However, several studies have reported larger increases in bifidobacteria following consumption of β2-1 fructans[5–7,29]. The increase in bifidobacteria seen in the present study may be smaller in comparison with that seen in some other studies because of differences in the prebiotic dose used, the duration of the intervention, type of β2-1 fructan used, the inherent characteristics of the subjects studied and the method used to measure the faecal bifidobacteria.

It was anticipated that Synergy1 would increase faecal bifidobacteria numbers, which was observed to occur, and that this effect would result in some modification of host immune parameters. A range of immune parameters was used in order to identify a potential effect on innate immunity, humoral immunity and T cell-mediated immunity. As a comparator, the digestible carbohydrate maltodextrin was used. No effects of Synergy1 were seen on any immune parameter measured, and it must be concluded that the prebiotic supplement had very little, if any, effect upon systemic immune function in healthy middle-aged adults. However, it is important to note that the data reported herein do not include any responses to exogenous immune stimulation, and these are likely to be more meaningful[31]. Previous studies in children[11,13], young adults[14], the elderly[2,16,19], adults with colon cancer[20,21], adults with active ulcerative colitis[22] and adults in intensive care[23] reported effects of β2-1 fructans on some aspects of immune function, but not on others, when examined in the absence of an exogenous immune challenge.

**Table 4.** Innate immune functions in subjects receiving placebo or prebiotic for 4 weeks

(Medians and 25th–75th percentiles)

| | Placebo group | | | | Prebiotic group | | | |
|---|---|---|---|---|---|---|---|---|
| | Week 0 | | Week 4 | | Week 0 | | Week 4 | |
| | Median | 25th–75th percentiles | Median | 25th–75th percentiles | Median | 25th–75th percentiles | Median | 25th–75th percentiles |
| Phagocytosis | | | | | | | | |
| Neutrophils (% positive)† | 99·1 | 98·1–99·5 | 92·9* | 82·6–96·3 | 99·1 | 94·4–99·5 | 92·2* | 82·7–95·5 |
| Neutrophils (MFI)† | 210 | 184–247 | 334* | 228–408 | 209 | 175–242 | 243* | 191–353 |
| Monocytes (% positive)† | 85·6 | 73·2–88·0 | 71·9* | 63·3–81·9 | 86·5 | 75·5–89·9 | 67·4* | 51·2–78·4 |
| Monocytes (MFI)† | 123 | 114–130 | 195* | 111–348 | 118 | 111–139 | 138 | 106–286 |
| Oxidative burst | | | | | | | | |
| Neutrophils (% positive)† | 97·2 | 87·3–98·9 | 96·9 | 93·6–98·6 | 97·9 | 95·9–98·5 | 97·1 | 89·9–98·1 |
| Neutrophils (MFI)† | 86 | 75–123 | 116* | 101–134 | 92 | 75–158 | 99 | 73 –133 |
| Monocytes (% positive)† | 79·4 | 74·1–85·1 | 76·8 | 67·6–83·6 | 79·8 | 72·9–85·8 | 76·4 | 67·2–82·6 |
| Monocytes (MFI)† | 23 | 20–30 | 27* | 23–31 | 24 | 21–28 | 24 | 22–29 |
| Neutrophils (% positive)‡ | 98·9 | 98·3–99·6 | 99·1 | 98·3–99·7 | 99·4 | 98·4–99·8 | 99·6 | 99·2–99·9 |
| Neutrophils (MFI)‡ | 281 | 149–361 | 303 | 220–385 | 263 | 222–361 | 280 | 197–379 |
| Monocytes (% positive)‡ | 98·9 | 93·2–99·9 | 95·3 | 91·3–99·8 | 98·4 | 93·9–99·6 | 97·7 | 87·5–99·6 |
| Monocytes (MFI)‡ | 37 | 25–48 | 33 | 25–43 | 31 | 27–42 | 33 | 27–49 |
| Natural killer cell activity specific cell lysis | | | | | | | | |
| E:T 100:1 | 15·1 | 8·7–19·2 | 17·4 | 12·9–20·9 | 14·9 | 10·5–22·7 | 12·3 | 7·1–18·1 |
| E:T 50:1 | 10·0 | 7·6–12·5 | 12·2 | 6·3–15·2 | 10·4 | 7·7–14·2 | 9·5 | 5·8–11·6 |
| E:T 25:1 | 6·9 | 4·0–7·4 | 6·2 | 4·0–11·2 | 7·1 | 5·8–11·2 | 7·3 | 4·3–10·7 |
| E:T 12·5:1 | 3·6 | 3·0–4·7 | 4·8 | 3·3–9·3 | 5·3 | 3·7–8·2 | 4·1 | 2·6–8·4 |
| E:T 100:1§ | 34·1 | 25·3–39·3 | 36·8 | 26·2–46·6 | 31·2 | 20·6–50·3 | 23·4 | 15·3–33·9 |
| E:T 50:1§ | 29·1 | 18·8–34·5 | 25·9 | 15·2–34·0 | 22·1 | 11·4–42·7 | 16·2 | 6·9–27·5 |
| E:T 25:1§ | 17·5 | 12·0–20·2 | 12·8 | 9·3–16·5 | 22·4 | 6·5–26·8 | 10·1 | 4·4–39·7 |
| E:T 12·5:1§ | 7·7 | 4·9–11·5 | 9·4 | 4·1–13·8 | 5·9 | 4·7–6·6 | 5·1 | 3·2–10·2 |

MFI, median fluorescence intensity; E:T, effector:target cell ratio.
* Significantly different from week 0 (Wilcoxon signed-rank test) ($P<0·05$).
† In response to *Escherichia coli*.
‡ In response to phorbol myristyl acetate.
§ Effector cells pretreated with IL-2.



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**Table 5.** Serum Ig and salivary IgA concentrations in subjects receiving placebo or prebiotic for 4 weeks (Medians and 25th–75th percentiles))

| | Placebo group | | | | Prebiotic group | | | |
|---|---|---|---|---|---|---|---|---|
| | Week 0 | | Week 4 | | Week 0 | | Week 4 | |
| Serum IgA (mg/ml) | 2·80 | 1·64–4·07 | 2·07 | 1·10–3·89 | 3·74 | 2·82–4·34 | 2·29* | 1·53–3·92 |
| Serum IgM (mg/ml) | 0·96 | 0·73–1·44 | 0·90* | 0·40–1·07 | 0·98 | 0·79–1·38 | 0·88* | 0·43–1·01 |
| Serum IgG (mg/ml) | 16·34 | 12·04–30·19 | 27·38* | 14·69–45·40 | 20·43 | 10·37–30·98 | 28·17 | 12·91–38·42 |
| Salivary sIgA (μg/μg protein) | 191·3 | 107·1–316·6 | 192·3 | 121·3–480·2 | 189·6 | 129·9–254·7 | 272·5 | 122·1–412·5 |
| Salivary sIgA (μg/ml) | 257·6 | 185·5–324·0 | 233·4 | 160·7–316·6 | 187·2 | 134·6–279·8 | 180·3 | 128·7–279·9 |

sIgA, secretory IgA.
* Median values were significantly different from week 0 (Wilcoxon signed-rank test) ($P<0·05$).

Since bifidobacteria counts were shown to increase over the 4-week time period, the lack of effect of Synergy1 on immune function cannot be due to an absence of a prebiotic effect. Several explanations could be offered for the lack of effect of Synergy1 upon systemic immune function in the present study. First, it is possible that the immune system is already functioning optimally in healthy middle-aged adults; the immune system of infants and the elderly may be more sensitive to the effects of prebiotics. Second, the dose of prebiotic may be important, with greater effects probably at higher doses. However, the dose of 8 g/d used in the present study is comparable with that used in other studies, which range from 0·88 to 12 g/d. Third, the type of β2-1 fructan used may influence the biological effects seen. As β2-1 fructans with different chain lengths are fermented in different parts of the intestine, this may influence any effect they may have on immune function. However, as Synergy1 contains both long-chain inulin and shorter-chain oligofructose, it would be expected to have an effect over different parts of the intestine. Fourth, the sample size of the present study, although comparable with many other studies of this type, is fairly small, and therefore, in a bigger study, significant effects may have been observed. Immune markers vary widely between individuals, and it may be that for some parameters measured, the sample size was not large enough to detect differences between the groups. Fifth, it is also possible that the change in faecal bifidobacteria seen was not large enough to influence host immune function; the extent of the bifidogenic effect will depend upon dose and duration of treatment, the chemical composition of the prebiotic, the age, health status and diet of the subjects. Sixth, bacteria other than bifidobacteria, such as lactobacilli, may be involved in the effect of fructo-oligosaccharides on the host immune system, and numbers of these bacteria may not have been affected in the present study. However, bacteria other than bifidobacteria were not assessed here, and so it is not possible

**Table 6.** Measures of T cell function in subjects receiving placebo or prebiotic for 4 weeks (Medians and 25th–75th percentiles)

| | | Placebo group | | | | Prebiotic group | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ConA | Week 0 | | Week 4 | | Week 0 | | Week 4 | |
| T cell activation | | | | | | | | | |
| $CD3^+CD4^+CD69^+$(%) | − | 11·0 | 8·8–18·3 | 11·7 | 7·9–18·3 | 18·7 | 10·7–24·4 | 12·3 | 8·3–20·1 |
| CD69 MFI | − | 42 | 36–47 | 44 | 36–53 | 42 | 39–47 | 43 | 39–49 |
| $CD3^+CD4^+CD69^+$(%) | + | 61·6 | 56·0–72·6 | 57·3 | 48·8–71·2 | 64·8 | 56·8–69·3 | 55·9* | 52·8–65·1 |
| CD69 MFI | + | 101 | 74–125 | 94 | 73–112 | 96 | 77–113 | 87 | 75–113 |
| T cell proliferation | | | | | | | | | |
| Proliferating cells (%) | − | 8·3 | 5·5–15·7 | 8·8 | 4·9–19·0 | 7·5 | 4·1–15·7 | 8·6 | 5·5–14·9 |
| CFSE MFI | − | 754 | 581–1086 | 785 | 633–1068 | 783 | 663–1033 | 787 | 666–936 |
| Proliferating cells (%) | + | 79·6 | 67·7–85·9 | 78·1 | 62·9–83·3 | 77·3 | 71·5–88·5 | 75·5 | 67·4–86·6 |
| CFSE MFI | + | 160 | 116–210 | 167 | 132–217 | 147 | 112–188 | 162 | 116–195 |
| Cytokine concentration (pg/ml) | | | | | | | | | |
| IL-2 | − | 1·6 | 1·3–2·1 | 1·6 | 1·3–2·1 | 1·8 | 1·3–2·7 | 1·8 | 1·3–2·2 |
| IL-4 | − | 1·5 | 1·3–2·1 | 1·8 | 1·3–2·5 | 1·3 | 1·3–1·7 | 1·3 | 1·3–1·6 |
| IL-6 | − | 1754 | 687–3294 | 2275 | 724–3857 | 1103 | 484–5103 | 1327 | 518–3390 |
| IL-10 | − | 12·7 | 9·4–20·7 | 18·2 | 5·8–21·1 | 14·8 | 8·1–29·7 | 12·1 | 7·9–19·0 |
| TNF-α | − | 29·0 | 10·1–47·6 | 30·2 | 10·7–57·7 | 36·7 | 4·6–50·9 | 23·3* | 9·4–32·9 |
| IFN-γ | − | 3·6 | 3·6–3·9 | 3·6 | 3·6–8·6 | 3·6 | 3·6–4·7 | 3·6 | 3·6–4·4 |
| IL-2 | + | 76·3 | 52·8–152·4 | 65·0 | 45·2–127·4 | 79·5 | 60·7–177·5 | 84·0 | 58·8–154·5 |
| IL-4 | + | 11·7 | 7·6–17·9 | 9·8 | 6·1–15·7 | 8·6 | 5·0–9·9 | 8·2 | 5·5–10·7 |
| IL-6 | + | 9451 | 4591–11 045 | 7004 | 1978–12 521 | 7764 | 3892–13 537 | 7771 | 6387–10 976 |
| IL-10 | + | 76·0 | 51·0–116·8 | 68·3 | 45·1–99·8 | 72·2 | 47·5–88·7 | 63·5 | 55·6– 119·4 |
| TNF-α | + | 382·5 | 220·1–544·4 | 292·3 | 152·1–477·9 | 306·4 | 164·6–574·3 | 360·7 | 89·2–566·9 |
| IFN-γ | + | 892 | 387–1555 | 713 | 293–1479 | 1236 | 397–2314 | 1210 | 519–2069 |

ConA, concanavalin A; MFI, median fluorescence intensity; CFSE, carboxyfluorescein succinimidyl ester; IFN, interferon.
* Median values were significantly different from week 0 (Wilcoxon signed-rank test) ($P<0·05$).

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to consider this possibility further as an explanation for the lack of immune effects seen. Finally, Synergy1 may alter aspects of the gut-associated immune system. However, no markers of immune function in the gut were measured in the present study that focused upon systemic markers measured in the blood, which may not reflect actions at the gut mucosa. The present study did measure salivary sIgA, but this is more representative of the upper respiratory tract immune system than the gut-associated immune system. Future studies could investigate markers of the gut-associated immune response where these are available. Faecal sIgA has been shown to increase in newborn infants supplemented with a combination of galacto-oligosaccharides and shorter-chain oligofructose[11,13]. It is also worth noting that a protective effect of β2-1 fructans may occur via an alteration in the immunological challenges imposed by a healthier gut microbiota rather than through measurable changes in immune parameters.

Studies have reported a difference in gut microbiota between normal-weight and obese individuals[32,33]. Furthermore, obesity is now recognised as a state of chronic low-grade inflammation characterised by elevated circulating concentrations of inflammatory markers including cytokines such as IL-6[34]. It is proposed that there is a link between the altered gut microbiota and the low-grade inflammation seen in obesity[35]. The present study recruited subjects across a BMI range extending from normal weight, through overweight and into obesity. A *post hoc* exploration of the relationship between BMI and faecal microbiota indicated a trend towards a lower percentage of bifidobacteria with increasing BMI, but there were few relationships between BMI and the immune parameters measured. However, the present study did not measure circulating concentrations of inflammatory markers. In addition, BMI was found not to result in different effects of Synergy1 on faecal bifidobacteria or the immune functions measured.

In conclusion, the present study shows that consumption of Synergy1 at 8 g/d for 4 weeks increases faecal bifidobacteria and improves regularity of bowel movements in healthy middle-aged adults, effects associated with increased flatulence. However, there are no alterations in a number of markers of systemic innate, humoral and T cell-mediated immunity measured in the absence of an *in vivo* immune challenge.

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