Xylo-oligosaccharides alone or in synbiotic combination with *Bifidobacterium* *animalis* subsp. l*actis* induce bifidogenesis and modulate markers of immune function in healthy adults: a double-blind, placebo controlled, randomized, factorial cross-over study

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**Abbreviations:**

Bi-07, *Bifidobacterium animalis* subsp *lactis;* BMI, body mass index; FISH, fluorescence in situ hybridisation; FOS, fructo-oligosaccharide; GALT, gut-associated lymphoid tissue; IFN-γ, interferon-γ; MDX, maltodextrin; NK, natural killer cell; NKT, natural killer T cell; PAMP, pathogen-associated molecular pattern, PBS, phosphate buffered saline; qPCR, quantitative polymerase chain reaction; SCFA, short chain fatty acid; Th, T helper cell; XOS, xylo-oligosaccharide.

**Abstract**

Prebiotics, probiotics and synbiotics are dietary ingredients with the potential to influence health and mucosal and systemic immune function by altering the gut microbiota composition. A candidate prebiotic (xylo-oligosaccharide, XOS, 8g/day), probiotic (*Bifidobacterium animalis* subsp *lactis* Bi-07, 109 CFU/day) or synbiotic (8g XOS + 109 CFU Bi-07/day) was provided to healthy adults (25-65yr) for 21d. The aim was to identify their effect upon bowel habit, self-reported mood, gut microbiota composition, blood lipids and immune function. XOS increased mean bowel movements per day (p=0.009), but did not alter symptoms of bloating, abdominal pain or flatulence, or the incidence of any reported adverse events compared to maltodextrin control. XOS significantly increased participant reported vitality (p=0.003) and happiness (p=0.034). XOS+Bi-07 was associated with lowest reported use of analgesics (p=0.004). XOS significantly increased faecal bifidobacterial counts (p=0.008) and fasting plasma HDL (p=0.005). Bi-07 significantly increased faecal *B. lactis* content (p=0.007), lowered lipopolysaccharide stimulated IL-4 secretion in whole blood cultures (p=0.035) and the IgA content of saliva (p=0.040) and increased IL-6 secretion (p=0.009). XOS resulted in lower expression of CD16/56 on NKT cells (p=0.027) and lower IL-10 secretion (p=0.049), while XOS and Bi-07 were associated with reduced CD19 expression on B cells (XOS\*Bi-07, p=0.009). This study demonstrates that XOS induces bifidogenesis, improves aspects of the plasma lipid profile and modulates markers of immune function in healthy adults. Provision of XOS+Bi-07 as a synbiotic may confer further advantages due to the discrete effects observed of Bi-07 upon the gut microbiota and markers of immune function.**Introduction**

Prebiotics are ‘a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health’ (1). Xylo-oligosaccharides (XOS) are relatively stable under acidic conditions, which may endow protection from digestion when passing through the stomach (2). Degradation of xylobiose (XOS dp = 2) in the intestine has been studied *in vitro* suggesting that XOS may be non-digestible and would reach the colon intact after oral intake (3).

The preferential fermentation of XOS by bifidobacteria, including *B. lactis* has been demonstrated *in vitro* using pure culture studies (4-9) and faecal batch and semi-continuous mixed culture fermentations (10-12). Animal studies demonstrate that XOS stimulates the growth of caecal and faecal bifidobacteria at higher levels than the prebiotic fructo-oligosaccharide (FOS) (13-15). In human studies, 2 – 5 g of XOS per day results in significant increases in faecal bifidobacteria; increased faecal short chain fatty acids (SCFA) concentrations and concomitant decreases in pH, proteolytic metabolites and enzyme activity (16-18) and has an ameliorating effect on constipation in women (19, 20). The evidence for the prebiotic capacity and health benefits of XOS is therefore promising, but further placebo-controlled studies are required, in particular to evaluate a wide range of health parameters (21, 22).

Prebiotics may influence immune function by altering the profile of pathogen-associated molecular patterns (PAMPs) presented to the gut-associated lymphoid tissue (GALT), or via indirect effects of microbial metabolic products, such as SCFA (23). Dietary XOS significantly increased resistance to *Listeria monocytogenes* in a guinea pig model (24), with *in vitro* studies confirming that XOS decreases the adherence of this pathogen to intestinal epithelial cells (25). A human study providing 5g / day XOS identified a bifidogenic effect, but no significant effects upon the markers of immune function measured (LPS induced cytokine production and faecal secretory IgA) were observed (18).

Positive effects of probiotics upon immune function have been observed in animal studies, and trials in children, adults and the elderly, but influences are species and strain specific and variable (26). Placebo-controlled studies using *Bifidobacterium animalis* subsp *lactis* (Bi-07) identified reduced incidence of bloating in adults with functional bowel disorders (27) and cold and influenza-like symptoms in children (28).

Provision of synbiotics (prebiotics combined with a probiotic) may increase probiotic survival after consumption and ensure persistence of the probiotic strain within the gut microbiota (29). However, studies using synbiotic preparations often have the limitation that it remains unclear whether any effect observed is a result of the prebiotic, probiotic or a synergistic effect of the combined supplement.

This double-blind, placebo controlled cross-over study investigates the effect of XOS (provided as a prebiotic and/or in combination with Bi-07) in healthy adults. It is our hypothesis that provision of XOS in combination with Bi-07 will be advantageous over individual provision of these supplements, due to a synergistic effect arising from the preferential fermentation of XOS by *B. lactis* (9), and that beneficial effects will include changes to the gut and immune function. The primary outcome measure is the effect of supplements upon the bifidobacteria content of faeces. Secondary outcome measures include the effect of supplements upon the gut microbiota and faecal short chain fatty acid content, bowel-habits, self-reported mood, plasma lipids and markers of immune function. Assessment of immune function is complex, requiring multiple markers to be monitored in order to fully assess the potential impact of a nutritional intervention. In this study, markers selected for analysis include those reflecting systemic and mucosal immunity, innate and acquired immunity, cellular activity and concentrations of soluble mediators.

**Experimental methods**

*Subjects*

The study group size required was estimated using G\*Power 3.0.10(30). On the basis of 5% significance, power of 95% and 0.5 correlation between groups for dependent means, a sample size of 7 would be required to detect an effect of XOS supplementation on the primary outcome (increased *Bifidobacterium* population within faeces (17)). Data from human studies which looked at the effects of probiotics (31,32), prebiotics (33) or synbiotics (34) upon immune function were used to determine the sample size required to detect significant effects upon immune function including markers of phagocytosis, changes to lymphocyte subsets and cytokine production. The effect sizes observed in these available human studies ranged from 0.3 - 1.7, with a variance of 0.2. On the basis of 5% significance, power of 95% and 0.5 correlation between groups for dependent means, the median sample size required for nine assessed immune outcomes was 27, with 6/9 outcomes requiring a n < 35. It was therefore aimed for 40 volunteers to complete the study, with 44 volunteers recruited to allow for participant drop-out, in order to allow significant effects upon both primary and secondary outcomes to be assessed. The minimum detectable effect size will therefore be 0.58 (on the basis of 5% significance, power of 95%).

This study was conducted according to guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Ethics and Research Committee of the University of Reading. Written informed consent was obtained from all subjects. Verbal consent was witnessed and formally recorded. In total, 44 volunteers were recruited from the Reading area between September 2008 and January 2009, and the study was completed in June 2009. Inclusion criteria were: a signed consent form, age 25-65y, body mass index (BMI) 20-30, and good general health, as determined by medical questionnaire. Exclusion criteria included: evidence of physical or mental disease requiring inpatient/outpatient treatment and/or use of prescription medication; planned major surgery; history of drug or alcohol abuse; severe allergies or a history of severe abnormal drug reaction; participation in experimental drug trial within four weeks prior to study; participation in prebiotic or laxative trials within the previous three months; use of antibiotics within the previous six months; chronic constipation, diarrhoea or other chronic gastro-intestinal complaint; intake of other prebiotics or probiotics within the previous four weeks, drugs active on gastrointestinal motility, or a laxative of any class for four weeks prior to study; use of prescribed medication; regular use of aspirin or other anti-inflammatory drugs.

*Study design*

Volunteers took dietary supplements in this double-blind, placebo-controlled, randomized, factorial cross-over study design (Clinicaltrials.gov identifier: NCT01545219). Supplements were provided for 21 days, with a 28 day washout period. The supplements were: XOS (8 g / day, Shandong Long-live Biotech, China), *B. lactis* (Bi-07 ATCC SD5220, 109 CFU / day, Danisco, Madison, USA), and the control, maltodextrin (Syral, Marckolsheim, France). Maltodextrin (MDX) was selected for use as the ‘placebo control’ as this carbohydrate is fully absorbed as glucose within the small intestine, and therefore will not influence the gut microbiota. Volunteers were provided with two sachets of daily supplements in powder form, and advised to dissolve the contents together in water, milk or fruit juice. In this double blind study, all supplements were identically packaged, and identified by alphabetic code. Volunteers were asked to refrain from consuming any other probiotic or prebiotic products during the study period, and given advice on common dietary products containing supplemental probiotics or prebiotics. Volunteers were provided with an excess of sachets at the beginning of each supplement period, and were asked to return unused sachets as a marker of compliance. Volunteers were randomised by gender, age and BMI to their starting point in the supplement sequence (MDX + MDX, XOS + MDX, Bi-07 + MDX, XOS + Bi-07) by covariate adaptive randomization (35). Staff responsible for enrollment of participants, assigning participants to supplement sequence and assessing outcomes remained blinded to treatment identity until data analysis was completed. Prior to starting the study, volunteers completed a 4-day food diary, which was analyzed using DietPlan6.60b (Forestfield Software Ltd, Australia). Volunteers attended study appointments before and after each supplement or washout period. At study appointments, anthropometric measurements were recorded (weight, blood pressure, waist circumference), volunteers provided a fasted blood sample, and samples of saliva and faeces were collected.

*Faecal sample processing*

A freshly voided faecal sample was collected in a sterile plastic pot at the start and end of each treatment and washout period. Samples were taken for use in faecal dry weight, and IgA assays were stored at -20 oC, and samples for quantitative polymerase chain reaction (qPCR) analyses and enumeration of total bacteria by flow cytometry stored at -80 oC. Remaining faecal samples were diluted 1 in 10 (wt : wt) in phosphate-buffered saline [(PBS); 0.1 M; pH 7.0] and homogenised in a Stomacher 400 (Seward, Norfolk, United Kingdom) for 2 min at normal speed (460 paddle beats / min). A 15 ml sample of faecal slurry was vortexed with 2 g of 3 mm diameter glass beads (VWR, Lutterworth, UK) and then centrifuged to remove particulate matter (1500 g, 2 min). Supernatant was collected for use in SCFA analysis and assessment of genus-level changes in the gut microbiota by fluorescence *in situ* hybridisation (FISH) with 16S rRNA targeted oligonucleotide probes.

*Fluorescence in situ hybridisation*

Faecal slurry supernatant was fixed in paraformaldehyde [1 : 4 v / v in 4% PFA in 0.1 M PBS, pH 7.2] for 4 hours at 4 oC, centrifuged (13,000 g 5 min), washed twice with 0.1 M PBS, resuspended in 1 : 1 PBS : Ethanol and stored at -20 oC. Oligonucleotide probes used were Cy-3 labeled and synthesized by Sigma-Aldrich (Poole, UK). Probes used were Bif164, Bac303, Chis150, Lab158 and ATO291 specific for *Bifidobacterium* spp.*, Bacteroides/Prevotella* group*, Clostridium* clusters I and II (including *C. perfringens* and *C. histolyticum*)*, Lactobacillus/Enterococcus* subgroup and *Atopobium* respectively.Samples were hybridized as described (36). Data are expressed as log10 counts / g dry weight faeces.

*Quantification of Bifidobacterium lactis*

DNA was extracted from the faecal samples with the use of the QIAampDNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer'sinstructions. Quantitative polymerase chain reaction (qPCR) was used for quantification of *B. lactis* using the FAST SYBR green methodology (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl containing 1 ng of template DNA and 250 nM of the forward primer Blact\_1 (12) and reverse primer Bflact5 (37). The amplification and detection of DNA were performed with an ABI 7500 sequencing detection system (Applied Biosystems). To obtain a standard curve, a 10-fold dilution series ranging from 10 pg to 10 ng of DNA from the bacterial standard culture of *B. lactis* (ATCC SD5220) was included in the PCR assays. For determination of DNA, triplicate samples were used, and the mean quantity per g dry weight was calculated.

*Quantification of total bacteria by flow cytometry*

Total bacteria in faeces was determined by using a flow cytometric FACSCalibur-system (BD Biosciences, San Jose, CA, USA) as previously described (38). Frozen faecal samples were thawed, and bacteria from the faecal samples were recovered by diluting and washing the samples 1:30 with washing buffer (50 mM sodium phosphate buffer, pH 8) on a reciprocating horizontal platform shaker at 200 rpm for 10 min, then centrifuged at 30,000 × *g* for 30 min at room temperature. The supernatant was discarded, and the pellet was washed three more times as described above. A subsample from the suspension was withdrawn before the last centrifugation, fixed with 4% formaldehyde and stained with a fluorescent, nucleic acid binding dye, SYTO 24 (Molecular Probes, Leiden, the Netherlands). The total number of bacterial cells was determined by comparing the cellular events to bead events within BD TrucountTM tubes (BD Biosciences, Oxford, UK).

*Short chain fatty acids*

Faecal slurry supernatant was used to determine faecal concentrations of SCFA including acetic acid, propionic acid, i-butyric acid, n-butyric acid, i-valeric acid, n-valeric acid, n-caproic acid and d/l-lactic acid by gas chromatography (39). Thawed faecal slurry supernatants were centrifuged at 13000 *g* for 10 min. 25 μl of internal standard (100 mM of 2-ethyl butyric acid), 25 μl of concentrated HCl and 1 ml of diethyl ether were added to 0.5 ml aliquots of centrifuged faecal slurry supernatants and centrifuged at 3000 *g* for 10 min. 400 μl of the resulting upper ether layer was combined with 50 μl of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide, heated at 80 ºC for 20 min in a water bath and then left at room temperature for 48 h to allow derivatization.

Samples were run on a 5890 series II Hewlett Packard GC system (HP, Crawley, UK) using dimethyl polysiloxane column (10m  0.18mm  0.20 μm df; Thomas Restek, Saunderton, UK) and detected with flame ionisation detector. Helium was used as carrier gas and sample (1 μl) was injected with a run time of 15 min. Detector was set to 275 ºC and the temperature of the oven was held at 60 ºC for 3 min, then increased 10 ºC per min to 150 ºC and held for 3 min. Chemstation REV.A.10.01 software (Agilent Technologies, West Lothian, UK) was used to integrate peaks. The concentrations of SCFA were determined by comparing their peak areas with external standards, relative to internal standard. Fatty acids concentrations were expressed as μmol / g of wet faeces.

*Volunteer bowel habit and mood questionnaires*

During supplement and washout periods, volunteers were provided with a daily diary to record their bowel habits and mood, use of medication or adverse events. Volunteers recorded the number of bowel movements per day, Bristol stool score and any symptoms of bloating or abdominal discomfort (rated as none, mild, moderate or severe)(40). Volunteers were asked to rate their flatulence on a 5-point scale (with a score of 0 for ‘normal’ and +1 or +2 for more than usual, -1 and -2 for less than usual). Self-reported mood was also assessed using a 5-point scale with vitality, stress, happiness and alertness recorded. Mean reported scores and the percentage of days where ‘more than usual’ or ‘less than usual’ of any symptom were reported during each supplement period were used for statistical analysis.

*Plasma lipids*

Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and non-esterified fatty acids were quantified using an automated clinical chemistry analyser (Instrumentation Laboratory Ltd., Warrington, UK) using enzyme-based colorimetric kits supplied by Instrumentation Laboratory (Warrington, UK) and Alpha Laboratories (Eastleigh, UK) in accordance with manufacturer instructions.

*Faecal and salivary IgA*

Faecal and salivary IgA content was determined by ELISA (Immunodiagnostik, Germany) in accordance with manufacturer instruction.

*Phagocytosis and oxidative burst*

Phagocytosis and oxidative burst by monocytes and granulocytes was determined in fresh blood samples using PHAGOTEST ® and BURSTTEST ® (ORPEGEN Pharma, Heidelberg, Germany) in accordance with manufacturer instructions.

*Immune cell counts and phenotypes*

Total white blood cell counts were obtained using a Beckman Coulter Z1 automated cell counter and ZAP-OGLOBIN II Lytic reagent (Beckman Coulter, High Wycombe, UK). Immune cell phenotyping was undertaken on fresh blood using stains for CD3 FITC/CD4 PE, CD3 FITC/CD8 PE, CD3 FITC/CD16 PE+CD56 PE, and CD3 FITC/CD19 PE from BD Biosciences (Oxford, UK) in accordance with manufacturer instructions in order to identify T, T helper (Th), cytotoxic T, natural killer (NK), natural killer T (NKT) and B cells. Red blood cells were lysed using PharmLyse (BD Biosciences, Oxford, UK) and samples washed twice with a buffer solution (PBS, 1% BSA, 0.1% Sodium Azide) and resuspended in a fixing solution (2% paraformaldehyde in PBS) prior to analysis. 10,000 events were collected on a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK) and data analysed using Flowjo 7.6.5 (Ashland, OR, USA).

*LPS-stimulated cytokine production in whole blood cultures*

1 / 10 diluted whole blood was incubated for 24 hr in the presence of 1 μg / ml LPS (Sigma-Aldrich, Gillingham, UK). Culture supernatants were assessed for cytokine production using a T-helper (Th)1 / Th2 cytokine array (Bender MedSystems, Vienna, Austria) which includes interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, Il-5, IL-6, IL-8, IL-10, IL-12p70, tumour necrosis factor (TNF)-α and TNF-β.

*Concanavalin-A stimulated expression of CD69 on immune cells*

1 / 10 diluted whole blood was incubated for 24 hr in the presence of 50 μg / ml Concanavalin A (Sigma-Aldrich, Gillingham, UK). CD69 (PE) expression on CD3, CD4 and CD8 (FITC) positive cells were determined using stains from BD Biosciences (Oxford, UK) in accordance with manufacturer instructions. Red blood cells were lysed using PharmLyse (BD Biosciences, Oxford, UK) and samples washed twice with a buffer solution (PBS, 1% BSA, 0.1% sodium azide) and resuspended in a fixing solution (2% paraformaldehyde in PBS) prior to analysis. 10 000 events were collected on a FACSCalibur flow cytometer (BD Biosciences, Oxford, UK) and data analysed using Flowjo 7.6.5 (Ashland, OR, USA).

*Statistics*

For continuous measurements, change with supplement (Δ) was considered (i.e. post-supplement value – pre-supplement value). Based on the residual analysis, some variables were log10 transformed prior to analysis. Data were analyzed using linear mixed-effects models (2x2 factorial approach) in a repeated-measures manner having a random effect for the subject accounting for repeated measures, continuous covariates for BMI, age, and fiber intake, fixed effect terms for the presence/absence of the prebiotic and probiotic and their interaction (XOS, Bi-07, XOS \* Bi-07, a 2 x 2 factorial setting), time point and starting point in the supplement sequence. Where significant effects of XOS, Bi-07 or XOS \* Bi-07 were observed, pairwise posts hoc comparisons with MDX were carried out using contrasts, adjusted using a single-step algorithm. Where significant carry over effects were observed (14/48 variables for time point, 1/48 variables for starting point in the supplement sequence) only data from the first supplement period was included in the final analysis. The questionnaire data was analysed using Friedman test followed by Bonferroni corrected post-hoc test that was based on mean rank differences between the supplements. Statistical significance was determined as p < 0.05.

The analyses were conducted with R: A Language and Environment for Statistical Computing (version 2.14.2; R Development Core Team, Vienna, Austria). The linear models were computed using R package nlme: Linear and Nonlinear Mixed Effects Models (version 3.1-102; J. Pinheiro, D. Bates, S. DebRoy, D. Sarkar, and R Development Core Team, Vienna, Austria). The model contrasts were computed using R package multcomp version 1.2.12 (41).

**Results**

*Volunteer characteristics and compliance*

The characteristics of the volunteers recruited to the study are described (**Table 1**). Of the 44 volunteers who started the trial, 41 completed. The reasons for drop-out were pregnancy (♀ age 31), vasovagal reaction to blood sampling (♂ age 57) and adverse reaction reported to study product (♀ age 64, probiotic). The adverse reaction reported to the probiotic occurred during the first supplement period, with this volunteer reporting headache (days 1–7, 9, 11 and 12 of supplement period), abdominal pain, bloating and increased flatulence (day 6 – 12 of supplement); participation in the study was halted on day 12 of supplement.

There was good volunteer compliance with the study timetable, with 93% of volunteers achieving supplement times of 21 ± 1 day, and 80% achieving washout periods of 28 ± 1 day. At the end of each supplement period, volunteers were asked to return any unused sachets in order that supplement compliance could be estimated. Compliance was estimated at >90% among the 35 volunteers who returned unused sachet, with no significant differences between supplement groups. At the end of each supplement period, volunteers were asked if they experienced any aftertaste, feelings of fullness or difficulty with taking the supplement provided. There were no significant differences between supplements for aftertaste (reported by 14-29% of participants) or feelings of fullness (reported by 6-14% of participants). There was a significant supplement effect upon reported difficulty taking the product (p = 0.006), with 32% of participants claiming difficulty with Bi-07, compared to 6-17% reported on the other supplements, with product insolubility the predominant reported difficulty (22/26 entries noted under additional comments).

*Gut microbiota*

XOS had a significant effect upon the bifidobacteria content of faeces (p = 0.008) (**Table 2**). There was no significant effect of supplement upon total bacterial counts, or the other genus-level probes investigated (**Table 2**). Supplementation with Bi-07 had a significant effect upon the faecal content of this strain (p = 0.007) (**Table 2**), indicative of volunteer compliance with supplements. Data indicate an additive effect of provision of XOS with Bi-07 upon change to the bifidobacterial content of faeces, as post-hoc testing reveals that only the XOS+Bi-07 supplement significantly increased bifidobacteria content compared to placebo. However, data does not support a specific synergistic effect of XOS in combination with Bi-07, as levels of faecal *B. lactis* were not significantly higher when XOS+Bi-07 was provided compared to Bi-07 alone. No significant supplement effect was observed upon faecal dry weight (**Table 2**). Significant supplement effects were observed upon faecal SCFA content **(Table 2**). XOS + Bi-07 had a distinct effect upon faecal SCFA content when provided in synbiotic combination compared with when provided individually (**Table 2**). When XOS or Bi-07 were provided individually, a reduction in acetic and butyric content was apparent, which did not occur when XOS+Bi-07 was provided. In contrast, the iso-valeric content of faeces increased while on XOS+Bi-07 supplementation.

*Volunteer bowel habit and mood questionnaires*

A significant increase in the number of bowel movements per day was observed among volunteers during XOS supplementation compared to MDX (p = 0.004, **Table 3**). No other significant supplement effects upon bowel habits or measures of bowel discomfort were observed. XOS supplementation resulted in significantly higher average reported vitality scores (p = 0.001) and happiness scores (p = 0.02) compared to MDX (**Table 4**). XOS supplementation also resulted in a lower proportion of days where volunteers reported having less vitality than usual (p = 0.002), while Bi-07 supplementation resulted in a higher proportion of days where volunteers reported having less happiness than usual compared to those on XOS supplement (p = 0.02). No significant effect of supplements were observed upon self-reported measures of alertness or stress.

The self-reported incidence of medication use was significantly influenced by supplements (**Table 5**), but not duration of medication use (data not presented). Use of analgesics during XOS+Bi-07 supplementation was significantly lower than while on MDX (p = 0.012), with a similar trend during XOS supplementation (p = 0.06). Further studies will be required to identify the change to symptom incidence or severity which underpins this reduced use of analgesic medication, as data from our study does not suggest any changes to the incidence or duration of headache, gastro-intestinal discomfort/abdominal pain or cold/flu-like symptoms. The self-reported incidence of adverse events was not significantly influenced by supplement (**Table 4**), nor the duration of symptoms reported (data not presented), though it should be noted that one volunteer withdrew from the study due following a reported adverse reaction to the probiotic supplement.

*Plasma lipids*

Fasting HDL was significantly higher among those volunteers who received XOS supplementation (p = 0.002, **Figure 1**), with an associated trend for a lower total cholesterol : HDL cholesterol ratio (p = 0.06). No significant changes to any other plasma lipids were observed (**Online supplementary data** **Table A**).

*Immune parameters*

There were no significant supplement effects upon measures of phagocytosis or oxidative burst, or total numbers of white blood cells (data not presented). Among NKT cells, XOS was associated with significantly lower expression of the cell surface markers CD16/56 (p = 0.027, **Figure 2A**). In B cells, XOS and Bi-07 were associated with lower CD19 expression on B cells, which may be indicative of changes to B cell subsets (XOS \* Bi-07, p = 0.009, **Figure 2B**). No other significant changes to peripheral blood mononuclear cell phenotypes were observed (**Online supplementary data Table B**), though there was a strong trend for a XOS \* Bi-07 interaction on expression of CD3 upon T cells (p = 0.050). A lower expression of CD3 reduces the potential for T cell activation, but is also a normal response to antigen-induced T cell activation. There were no significant supplement effects observed upon expression of the activation marker CD69 on T cells after culture with the mitogen ConA (data not presented).

Supplementation with Bi-07 significantly lowered the IgA content of saliva (p = 0.04, **Figure 3**). Faecal IgA content was not significantly altered by supplement use (**Table 2**).

There were significant effects of supplements upon cytokine secretion of whole blood cultured *ex vivo* with LPS. Supplementation with Bi-07 was associated with low IL-4 production (Figure 4A, p = 0.035) and higher IL-6 production (Figure 4B, p = 0.009), while XOS was associated with lower IL-10 production (Figure 4C, p = 0.049). No significant supplement effects were observed upon the other cytokines assessed (**Online supplementary data C**).

**Discussion**

This placebo-controlled crossover study investigated the acceptability and efficacy of XOS supplementation in a European-based population. XOS provided at 8g/day for a 3 week period was well tolerated by healthy adults, with no significant effects upon reported symptoms of abdominal pain, bloating or flatulence. XOS significantly increased self-reported vitality and happiness scores, and the synbiotic combination of XOS + Bi-07 was associated with significantly lower use of analgesics during the supplementary period. It may be advantageous to provide XOS as a synbiotic with Bi-07, in order to simultaneously confer the benefits observed with Bi-07 supplementation, while optimising product acceptability, as solubility in particular was poor when Bi-07 was provided alone. This synbiotic preparation is therefore recommended due to additive rather than specific synergistic effects.

XOS supplement resulted in modest, but significant, increases in the bifidobacterial counts in faecal samples when provided as either a prebiotic or synbiotic, confirming the observations reported in studies of Japanese volunteers (16, 17). An increased bifidobacterial content of faeces may improve colonic health, by competing with potential pathogenic organisms and/or interacting with gut-associated lymphoid tissue (23). Data does not support an additive or synergistic effect of XOS + Bi-07 on the gut microbiota, as the increase in faecal *B. lactis* content observed with Bi-07 treatment was not further enhanced by co-administration of XOS, nor were the changes observed in bifidobacteria after XOS + Bi-07 supplementation significantly different from those observed with XOS alone. This study utilised FISH to characterise genus-level changes to the gut microbiota. In future studies, methods such as metagenomics could be utilised to more fully characterise changes within the microbiome, and may reveal further changes associated with these dietary supplements. For example, the effects observed upon faecal SCFA content during supplementation with XOS + Bi-07 may reflect a shift from carbohydrate fermentation towards catabolism of protein within the microbiota, which may be more fully characterised and explored using alternative techniques.

Volunteers who received XOS supplements had a significant increase in fasting plasma HDL content, with an associated trend for a lower total cholesterol : HDL cholesterol ratio. Lower plasma HDL content has been identified as a significant risk factor for coronary disease (42). The average 0.07mM increase in HDL observed in volunteers receiving XOS is modest, but a 0.1mM increase has been estimated to induce a 10% reduction in coronary heart disease risk (43). Proposed mechanisms for the influence of dietary probiotics and prebiotics upon plasma lipids have been generated based upon data from *in vitro* studies and animal models. These mechanisms include the effect of probiotic bacteria upon bile acids, the ability of probiotics to bind cholesterol, the influence of circulating SCFA upon hepatic cholesterol synthesis, the role of prebiotics in reducing cholesterol absorption, and effects of fermentable carbohydrates upon the rates of gastric emptying (44,45). In our data, no correlations were observed between changes to faecal SCFA content and plasma HDL (data not presented). Further studies will be required to identify the mechanisms by which XOS exerts this HDL-lowering effect, and whether changes to vascular function can be detected. However, selection of an appropriate ‘placebo-control’ is challenging for a study investigating probiotics and prebiotics. It is possible that the MDX control itself exerted effects upon parameters measured, and data indicates that changes occurring within the placebo group may be driving some of the treatment effects observed upon HDL cholesterol. This suggests that even a modest increase in daily sugar intake is sufficient to alter HDL cholesterol. In addition, only data from the first treatment period was included in the analysis due to carry-over effects observed upon this variable. Use of a completely counterbalanced study design, rather than the incomplete counterbalanced measures design may have mitigated the risk of carry-over, and increased the resulting power of this observation.

Effects of both XOS and Bi-07 were observed upon measures of immune status and function. The effects that both probiotics and prebiotics can exert upon immune function have been well described in a range of studies including in vitro assessment, animal models and human trials (26,46). Probiotics can exert indirect effects upon immunity, via mechanisms including: changes to the gut microbiota, competitive inhibition of potential pathogen binding sites and improved gut barrier function (46). Probiotics also directly influence signalling pathways in intestinal epithelial cells and dendritic cells, with the potential to induce downstream effects upon immune function (47). Prebiotics may influence immune function indirectly, by altering the composition of the gut microbiota, or via their own direct effects, such as changes to PAMPs presented to the GALT (23). This study does not enable conclusions to be drawn about the mechanism of action of XOS and/or Bi-07, but instead provides information on the systemic and mucosal immune measures which have the potential for modification by supplementation. XOS induced changes to cell surface markers upon NKT cells and was associated with lower IL-10 secretion. However, it cannot be excluded that the effects observed upon NKT cells were in part influenced by the apparent increase in cell surface marker expression during MDX supplementation. Bi-07 had significant effects upon systemic markers of immune function, with lower IL-4 secretion and salivary IgA content and higher IL-6 secretion. A XOS \* Bi-07 interaction altered B cell surface marker expression. IL-4 secretion is associated with Th2-inflammatory conditions such as asthma and hay fever, and promotes B cell differentiation (47). NKT cells are an important link between innate and adaptive immunity. IL-6 and IL-10 are cytokines with pro- and anti-inflammatory actions, respectively, and the relative balance of these cytokines is important to prevent excessive inflammation. Salivary IgA is a marker of mucosal immunity, with secretion lowered during psychological and physical stress and lower levels associated with increased risk of urinary tract infections (48). Taken together, these effects suggest that XOS and Bi-07 have immunostimulatory effects, promoting Th1 responses and lowering Th2 activity. These effects may therefore be of benefit to individuals with supressed Th1 activity, e.g. elderly adults, or those with excessive Th2 activity, such as occurs in atopic disease. The functional consequences of these changes to measures of immune function should be investigated in suitably designed human studies, preferably using *in vivo* markers of immune function, such as the incidence of allergic rhinitis symptoms, or influenza vaccination responses.

The synbiotic XOS + Bi-07 is a suitable nutritional supplement for healthy adults, with increased reported vitality and happiness and reduced use of analgesics among participants. Data indicate potential benefits of both XOS and Bi-07, either as a result of their bifidogenic properties, increased fasting HDL and/or immunomodulatory activity. However, data does not support a specific synergistic effect of XOS + Bi-07 on the gut microbiota. Further studies will be required to confirm whether the effects of Bi-07 observed upon markers of immune function translate into alterations in functional or clinical measures of immune function such as the incidence of infection, allergic rhinitis or response to vaccination, or if providing a supplement containing XOS alone or in synbtiotic combination with Bi-07 can benefit those with gastrointestinal disorders or inflammatory conditions.

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**Conflict of Interest**

At the time of this study HR, SDF and ACO were employees of Active Nutrition, DuPont Nutrition & Health (formerly Danisco, Finland), which manufactures and markets the tested probiotic and sponsored the study. None of the other authors have a conflict of interest to report.

**Authorship**

GRG, KMT, RAR, ACO, CEC and PY designed research; CEC, AF, SDF, NH, YNL and CJS conducted research; CEC, HR, EA and SDF analyzed data; CEC, HR and SDF wrote paper. CEC had primary responsibility for final content. All authors read and approved the final manuscript.

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**Figure 1** Significant changes to fasted plasma HDL among volunteers recruited to a double-blind, placebo-controlled, randomized cross-over study of a candidate prebiotic (xylo-oligosaccharide, XOS, 8g/day), probiotic (*Bifidobacterium animalis* subsp *lactis*, Bi-07, 109 CFU/day) or synbiotic (XOS + Bi-07)1

1 Data is change from baseline, mean ± 95 % CI, (n = 11, data from first treatment period only). Mean baseline = 1.4 mM (SD 0.5). P values given are linear mixed-effects models (2x2 factorial approach) for the presence/absence of the prebiotic (XOS) and probiotic (Bi-07) and their interaction (XOS \* Bi-07). \* significantly different from maltodextrin, pairwise posts hoc comparisons carried out using contrasts, adjusted using a single-step algorithm.

**Figure 2** Significant changes to peripheral blood mononuclear cell phenotype among volunteers recruited to a double-blind, placebo-controlled, randomized cross-over study of a candidate prebiotic (xylo-oligosaccharide, XOS, 8g/day), probiotic (*Bifidobacterium animalis* subsp *lactis*, Bi-07, 109 CFU/day) or synbiotic (XOS + Bi-07)1

1 Data is change from baseline, mean ± 95 % CI, (n = 11, data from first treatment period only). **A**: Geometric mean fluorescence intensity (MFI) of CD16/56 on NKT cells, mean baseline = 32.2 (SD 8.9). **B**: Geometric mean fluorescence intensity (MFI) of CD19 on B cells, mean baseline = 180 (SD 36). P values given are linear mixed-effects models (2x2 factorial approach) for the presence/absence of the prebiotic (XOS) and probiotic (Bi-07) and their interaction (XOS \* Bi-07). \* significantly different from maltodextrin, pairwise posts hoc comparisons carried out using contrasts, adjusted using a single-step algorithm.

**Figure 3** Significant changes to salivary IgA content of volunteers recruited to a double-blind, placebo-controlled, randomized cross-over during supplementation with a candidate prebiotic (xylo-oligosaccharide, XOS, 8g/day), probiotic (*Bifidobacterium animalis* subsp *lactis*, Bi-07, 109 CFU/day) or synbiotic (XOS + Bi-07)1

1 Data is change from baseline, mean ± 95% CI (n = 41 - 42). Mean baseline = 1.4 mg / ml (SD 0.8). P values given are linear mixed-effects models (2x2 factorial approach) for the presence/absence of the prebiotic (XOS) and probiotic (Bi-07) and their interaction (XOS \* Bi-07).

**Figure 4** Significant changes to LPS stimulated cytokine production of whole blood cultures among volunteers recruited to a double-blind, placebo-controlled, randomized cross-over study of a candidate prebiotic (xylo-oligosaccharide, XOS, 8g/day), probiotic (*Bifidobacterium animalis* subsp *lactis*, Bi-07, 109 CFU/day) or synbiotic (XOS + Bi-07)1

1 Data is change from baseline, mean ± 95 % CI. **A**: IL-4, mean baseline = 3.7 pg / ml (SD 7.9), (n = 41 - 42). **B**: IL-6, mean baseline = 3.5 ng / ml (SD 3.2), (n = 11, data from first treatment period only). **C**: IL-10, mean baseline = 79.7 pg / ml (SD 88.8), (n = 11, data from first treatment period only). P values given are linear mixed-effects models (2x2 factorial approach) for the presence/absence of the prebiotic (XOS) and probiotic (Bi-07) and their interaction (XOS \* Bi-07)