

A prebiotic galactooligosaccharide mixture reduces severity of hyperpnoea-induced bronchoconstriction and markers of airway inflammation

Neil C. Williams^{1*}, Michael A. Johnson¹, Dominick E. Shaw², Ian Spendlove³, Jelena Vulevic⁴, Graham R. Sharpe¹ and Kirsty A. Hunter¹

¹Exercise and Health Research Group, Department of Sport Science, Sport, Health and Performance Enhancement (SHAPE) Research Centre, Nottingham Trent University, Nottingham NG11 8NS, UK

²Respiratory Research Unit, University of Nottingham, Nottingham NG5 1PB, UK

³Academic Department of Clinical Oncology, City Hospital Campus, University of Nottingham, Nottingham NG5 1PB, UK

⁴Clasado Research Services, Science and Technology Centre, Reading RG6 6UR, UK

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Abstract

Gut microbes have a substantial influence on systemic immune function and allergic sensitisation. Manipulation of the gut microbiome through prebiotics may provide a potential strategy to influence the immunopathology of asthma. This study investigated the effects of prebiotic Bimuno-galactooligosaccharide (B-GOS) supplementation on hyperpnoea-induced bronchoconstriction (HIB), a surrogate for exercise-induced bronchoconstriction, and airway inflammation. A total of ten adults with asthma and HIB and eight controls without asthma were randomised to receive 5.5 g/d of either B-GOS or placebo for 3 weeks separated by a 2-week washout period. The peak fall in forced expiratory volume in 1 s (FEV₁) following eucapnic voluntary hyperpnoea (EVH) defined HIB severity. Markers of airway inflammation were measured at baseline and after EVH. Pulmonary function remained unchanged in the control group. In the HIB group, the peak post-EVH fall in FEV₁ at day 0 (−880 (SD 480) ml) was unchanged after placebo, but was attenuated by 40% (−940 (SD 460) v. −570 (SD 310) ml, *P* = 0.004) after B-GOS. In the HIB group, B-GOS reduced baseline chemokine CC ligand 17 (399 (SD 140) v. 323 (SD 144) pg/ml, *P* = 0.005) and TNF-α (2.68 (SD 0.98) v. 2.18 (SD 0.59) pg/ml, *P* = 0.040) and abolished the EVH-induced 29% increase in TNF-α. Baseline C-reactive protein was reduced following B-GOS in HIB (2.46 (SD 1.14) v. 1.44 (SD 0.41) mg/l, *P* = 0.015) and control (2.16 (SD 1.02) v. 1.47 (SD 0.33) mg/l, *P* = 0.050) groups. Chemokine CC ligand 11 and fraction of exhaled nitric oxide remained unchanged. B-GOS supplementation attenuated airway hyper-responsiveness with concomitant reductions in markers of airway inflammation associated with HIB.

Key words: Asthma: Prebiotics: Airway inflammation: Bronchoconstriction: Gut microbiota

Asthma is a heterogeneous disease that affects approximately 235 million people worldwide⁽¹⁾. It is characterised by intermittent reversible bronchoconstriction, chronic airway inflammation and respiratory symptoms such as wheezing, dyspnoea, chest tightness and cough. The immunopathology of asthma is predominantly orchestrated by T-helper 2 (T_H2) cells and their pro-inflammatory cytokines and chemokines, which recruit secondary effector cells including IgE-activated mast cells, macrophages, basophils and eosinophils⁽²⁾. Exercise-induced bronchoconstriction (EIB) is a phenotype of asthma, which is characterised by transient airway narrowing during and/or after exercise^(3,4). EIB is ascribed to airway drying and changes in airway osmolality, which result in degranulation of inflammatory cells and release of inflammatory mediators⁽⁴⁾.

Inhaled corticosteroids and short- and long-acting β_2 -agonists provide effective therapy for asthma, but they are not curative nor do they modify disease progression⁽⁵⁾. Furthermore, long-term inhaled corticosteroid use has undesirable side-effects and adherence is poor, whereas chronic β_2 -agonist use results in tolerance⁽⁵⁾. The development of therapies that modulate the immunopathology of asthma without adverse side-effects is therefore desirable.

Gut microbes have a substantial influence on systemic immune function and allergic sensitisation^(6–8); thus, it is possible that manipulation of the gut microbiome may provide a potential strategy to influence the immunopathology of asthma. The observation that allergic asthma patients display lower levels of *Bifidobacterium adolescentis* supports this

Abbreviations: B-GOS, Bimuno-galactooligosaccharide; CCL11, chemokine CC ligand 11; CCL17, chemokine CC ligand 17; CRP, C-reactive protein; EIB, exercise-induced bronchoconstriction; EVH, eucapnic voluntary hyperpnoea; F_{ENO}, fraction of exhaled nitric oxide; FEV₁, forced expiratory volume in 1 s; HIB, hyperpnoea-induced bronchoconstriction; PEF, peak expiratory flow; T_H2, T-helper 2.

* **Corresponding author:** Dr N. C. Williams, email neil.williams@ntu.ac.uk

concept⁽⁹⁾. Moreover, in humans, 4 weeks of supplementation with *Bifidobacterium breve* M-16V combined with a prebiotic (non-digestible carbohydrate that enhances the growth and/or activity of beneficial indigenous gut bacteria) improved peak expiratory flow (PEF) and attenuated serum IL-5 after bronchial allergen challenge⁽¹⁰⁾.

Prebiotic galactooligosaccharides are derived from the action of the enzyme β -galactosidase. Bimuno-galactooligosaccharide (B-GOS) is especially potent in selectively increasing the growth and/or activity of bifidobacteria^(11–15), which has been shown to elicit beneficial immunomodulatory effects in both elderly and overweight adults^(12,14). In mice exposed to house dust mites, dietary galactooligosaccharides prevented the development of airway hyper-responsiveness and airway eosinophilia and reduced T_H2 -related cytokine IL-13 and chemokines (chemokine CC ligand 17 (CCL17), chemokine CC ligand 5 (CCL5)) in the lungs⁽¹⁶⁾. The effects of B-GOS on asthma in humans are currently unknown.

In humans, T_H2 -driven inflammation can be monitored using biomarkers such as the chemokine CC ligand 11 (CCL11) and CCL17, along with IgE, TNF- α , C-reactive protein (CRP) and fraction of exhaled nitric oxide ($F_{E}NO$)^(3,17). Eucapnic voluntary hyperpnoea (EVH) causes a highly reproducible hyperpnoea-induced bronchoconstriction (HIB) (a surrogate for EIB) in adults⁽¹⁸⁾, which makes this an excellent challenge test to evaluate the effects of B-GOS supplementation on airway hyper-responsiveness.

Therefore, the aim of this study was to test the hypothesis that B-GOS supplementation in adults with asthma attenuates the severity of HIB and that this is associated with reduced systemic concentrations of T_H2 -driven inflammatory markers.

Methods

Participants and study design

A total of ten participants (five males) formed a HIB group, and eight participants (five males) with no history of asthma formed a control group (Fig. 1; Table 1). All participants were non-smokers. Inclusion criteria for the HIB group were physician diagnosis of asthma, a baseline forced expiratory volume in 1 s (FEV_1) > 65% of the predicted⁽¹⁸⁾ and a $\geq 10\%$ fall in FEV_1 following an initial EVH screening test⁽¹⁹⁾. Participants in the HIB group were on steps 1–3 of the global initiative for asthma stepwise approach to asthma control⁽²⁰⁾.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Nottingham Trent University Human Ethics Committee (Approval no. 229; Clinical Trial no. ISRCTN15022880). All participants provided their written informed consent. The present study adopted a randomised (block randomisation), double-blind, placebo-controlled, cross-over design over 8 consecutive weeks. Participants were randomly assigned to receive 5.5 g/d of either B-GOS or placebo (maltodextrin) (Clasado Ltd) for 3 weeks. Thereafter, participants followed a 2-week washout period⁽¹¹⁾ (normal diet) before commencing the alternative supplement for the remaining 3 weeks (Fig. 1). The supplements were identical in taste and colour, and double blinding was completed by

Clasado Ltd. The B-GOS dose used in the present study has been shown to consistently increase the number of bifidobacteria within the gut^(11–15).

At day 0 and day 21 of each treatment, baseline $F_{E}NO$ was measured and an EVH test was performed at Nottingham Trent University. Before and after EVH, pulmonary function was assessed, and venous blood samples were collected for analysis of inflammatory markers. HIB participants were permitted to use their medication as required but stopped taking it before each EVH test (see below).

Fraction of exhaled nitric oxide, pulmonary function and eucapnic voluntary hyperpnoea test

Baseline $F_{E}NO$ was measured (NIOX MINO; Aerocrine) according to American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines⁽²¹⁾ in the HIB group only, as it is elevated in asthma patients but not in healthy controls⁽²²⁾. In accordance with ATS/ERS guidelines⁽²³⁾, pulmonary function (forced vital capacity (FVC), PEF and FEV_1) was assessed in triplicate at baseline and in duplicate at 3, 6, 10, 20 and 30 min after EVH. The highest values recorded were used for analyses. Whole blood (20 ml) was collected at baseline and 15 min, 60 min and 24 h after EVH. The EVH test comprised 6 min of EVH using a dry gas mixture at a target minute ventilation (\dot{V}_E) of 85% of the predicted maximum voluntary ventilation ($30 \times \text{baseline } FEV_1$)^(18,24). Participants avoided exercise for 24 h before each EVH test, and participants in the HIB group stopped taking their medication (inhaled corticosteroids: 4 d; inhaled long-acting β_2 agonists: 2 d; inhaled short-acting β_2 agonists: the day of the test)⁽²⁴⁾. On EVH test days, participants abstained from caffeine and alcohol and arrived at the laboratory > 2 h postprandially^(18,24).

Analysis of inflammatory markers

Concentrations of serum chemokines CCL11 and CCL17 were determined using multiplex analysis (Bio-Plex 200; Bio-Rad Laboratories Limited) and Luminex screening assay plates (R&D Systems) as previously described⁽²⁵⁾. ELISA was used to determine concentrations of TNF- α (R&D Systems), CRP and IgE (Universal Biologicals). The TNF- α ELISA does not cross-react with human IL-1 β , IL-1 α , IL-2-13 or TNF- β and is specific for the measurement of natural and recombinant human TNF- α . For TNF- α , the intra- and inter-assay variation was < 10% and the minimum detectable level of the assay was 0.60 pg/ml, which all samples exceeded. For CRP and IgE, the minimum detectable levels of the assays were 0.25 ng/ml and 0.29 KU/l, respectively, which all samples exceeded.

Statistics

The average minimum perceptible improvement in FEV_1 in adults with asthma is 230 ml⁽²⁶⁾, whereas the within-participant standard deviation for the fall in FEV_1 after EVH is 100 ml⁽¹⁸⁾. An *a priori* sample size calculation revealed that with power = 0.90 and $\alpha = 0.05$, a sample size of seven would be required to detect a 230-ml change in the fall in FEV_1 after EVH.

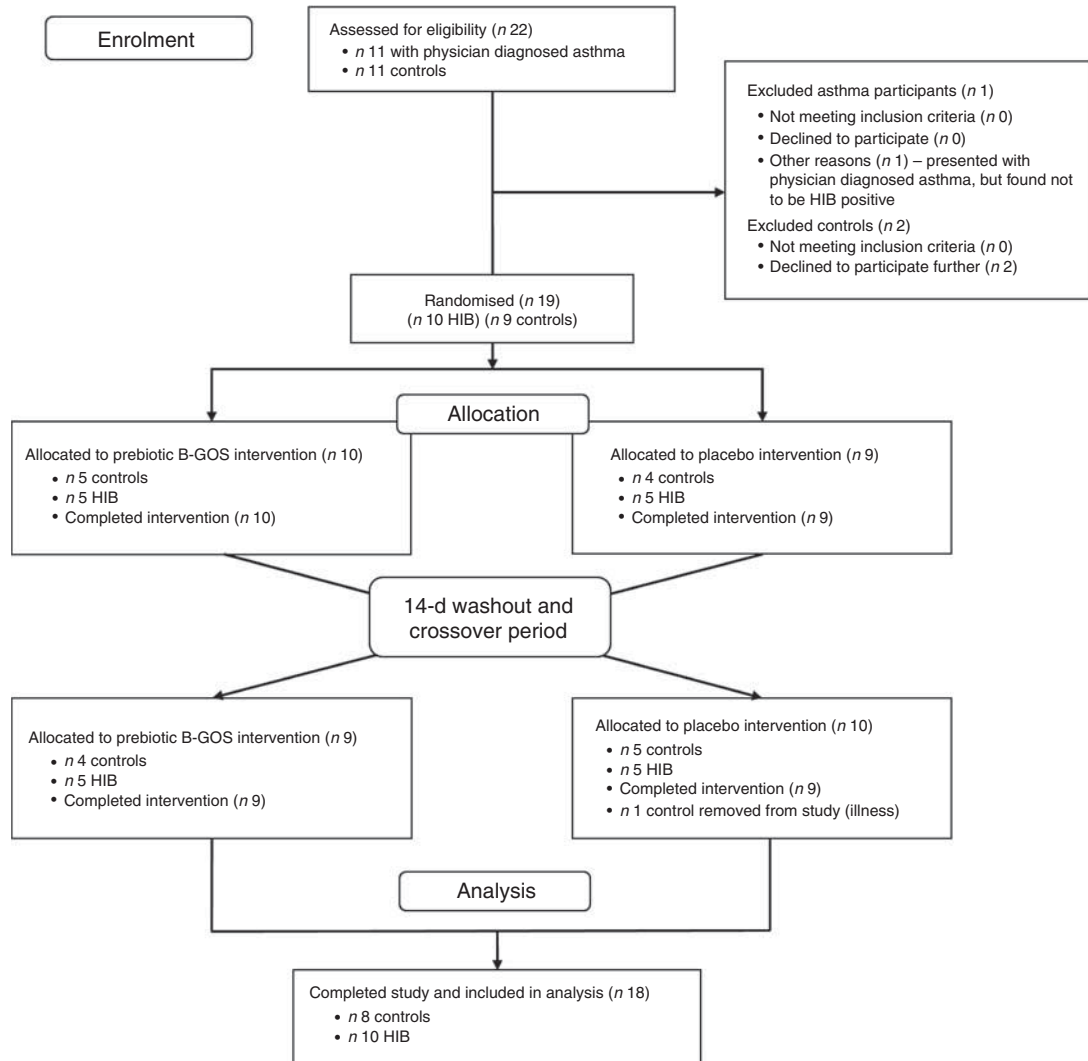


Fig. 1. Participant flow diagram. HIB, hyperpnoea-induced bronchoconstriction; B-GOS, Bimuno-galactooligosaccharides.

Pulmonary function data and serum TNF- α concentrations were analysed using repeated-measures ANOVA and Bonferroni-adjusted paired *t* tests. CCL11, CCL17, CRP and IgE were analysed using non-parametric Friedman's repeated measures and Wilcoxon's signed-rank tests. Between-group differences in IgE were analysed using the Kruskal–Wallis test. The AUC of the percentage fall in FEV₁ during the 30-min period after EVH (% Δ FEV₁AUC_{0–30}) was calculated using trapezoidal integration. Statistical significance was set at *P* < 0.05. Data are presented as mean values and standard deviations unless otherwise indicated.

Results

Pulmonary function

In the control and HIB groups, there were no between-day differences in baseline FEV₁. Pooled baseline FEV₁ tended to be lower in the HIB group compared with the control group (*P* = 0.066) (Table 1).

For the peak fall in FEV₁ after EVH, three-way, repeated-measures ANOVA revealed a treatment \times day \times group interaction (*P* < 0.001). In the control group, the peak fall in FEV₁ after EVH was unchanged after placebo and B-GOS (pooled data: –3 (SD 3) %). In the HIB group, the peak fall in FEV₁ after EVH was unchanged from day 0 (–880 (SD 480) ml) to day 21 (–840 (SD 430) ml) of placebo. Conversely, following B-GOS, the peak fall in FEV₁ after EVH was attenuated by 40 % from day 0 (–940 (SD 460) ml) to day 21 (–570 (SD 310) ml) (mean difference = 370 (SD 290) ml; 95 % CI 166, 575 ml, *P* = 0.004) (Fig. 2 and 3). In the control group, the overall severity of HIB, as determined by % Δ FEV₁AUC_{0–30}, was unchanged after placebo and B-GOS (pooled data: –48 (SD 53) ml). In the HIB group, % Δ FEV₁AUC_{0–30} was unchanged from day 0 (–530 (SD 384)) to day 21 (–523 (SD 366)) of placebo, whereas a 41 % reduction was observed from day 0 (–583 (SD 404)) to day 21 (–345 (SD 267)) of B-GOS (mean difference = –237 (SD 263); 95 % CI –425, –48, *P* = 0.019).

In the control and HIB groups, there were no between-day differences in baseline FVC and PEF. Baseline FVC and PEF

Table 1. Anthropometric data, baseline pulmonary function and medication (Numbers and percentages of predicted; mean values and standard deviations)

					FVC (litres)		FEV ₁ (litres)		PEF (litres/s)		
	Sex	Age (years)	Height (cm)	Body mass (kg)	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	Medications
HIB											
1	M	20	173	78	4.48	91	3.33	80	7.6	78	S
2	M	27	181	75	6.04	112	5.20	115	10.4	102	S
3	M	42	186	84	5.18	100	3.97	94	11.7	119	BUD, S
4	M	36	177	79	5.93	93	3.63	89	8.6	90	BUD/FORM, S
5	M	19	173	64	3.92	83	2.61	65	6.3	65	BUD/FORM, S
6	F	30	166	58	3.83	103	3.16	98	8.3	116	S
7	F	25	159	62	3.55	100	2.99	98	6.6	96	S
8	F	28	168	62	4.05	106	3.13	94	6.8	93	Sm/FORM, S
9	F	24	170	73	3.89	97	3.19	91	7.2	96	BEC, S
10	F	21	173	63	2.93	88	2.70	78	7.3	91	BUD, Sm
Mean (sd)		27 (7)	173 (8)	70 (9)	4.38 (1.03)	97 (9)	3.39 (0.75)	90 (14)	8.1 (1.6)	95 (16)	
Control											
1	M	25	181	77	5.21	96	3.78	83	9.8	96	
2	M	23	167	57	4.67	101	4.10	104	9.0	96	
3	M	27	177	68	5.06	101	4.29	101	11.5	113	
4	M	23	189	91	5.39	90	5.12	102	11.8	110	
5	M	31	183	80	5.26	97	4.06	90	8.2	82	
6	F	22	159	65	3.97	114	3.13	103	7.0	101	
7	F	32	169	58	4.04	107	2.94	90	6.8	95	
8	F	22	168	82	4.25	110	3.71	110	9.2	125	
Mean (sd)		26 (4)	174 (10)	72 (12)	4.73 (0.58)	102 (8)	3.89 (0.68)	98 (9)	9.2 (1.9)	102 (13)	

HIB, hyperpnoea-induced bronchoconstriction; FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s; PEF, peak expiratory flow; M, male; S, salbutamol; BUD, budesonide; /, in combination with; FORM, formoterol; F, female; Sm, salmeterol; BEC, beclomethasone.

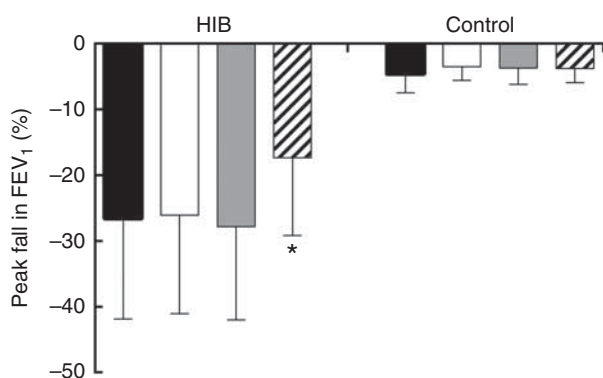


Fig. 2. Peak fall in forced expiratory volume in 1 s (FEV₁) after eucapnic voluntary hyperpnoea. Values are means and standard deviations represented by vertical bars. * Bimuno-galactooligosaccharides (B-GOS) day 0 v. B-GOS day 21 ($P=0.004$). ■, Placebo day 0; □, placebo day 21; ▒, B-GOS day 0; ▨, B-GOS day 21.

were not different between control and HIB groups (Table 1). The peak fall in FVC after EVH was greater in the HIB group (-18 (SD 16)%; -78 (SD 54)ml) than in the control group (-3 (SD 2)%; -14 (SD 12)ml) ($P=0.005$), but was unchanged in both groups after placebo and B-GOS. The peak fall in PEF after EVH was greater in the HIB group (-27 (SD 12)%) than in the control group (-9 (SD 6)%) ($P=0.007$) and was unchanged after placebo in both groups and after B-GOS in the control group. Conversely, after B-GOS in the HIB group, the peak fall in PEF after EVH was reduced from day 0 (-28 (SD 14)%) to day 21 (-17 (SD 10)%) (mean difference = 11 (SD 13)%; 95% CI 1.8, 20.9%, $P=0.024$).

Serum TNF- α

At day 0, baseline TNF- α was higher in the HIB group (2.64 (SD 0.81)pg/ml) than in the control group (1.37 (SD 0.37)pg/ml) ($P=0.001$). A four-way, repeated-measures ANOVA revealed an intervention \times day \times time \times group interaction ($P=0.036$). Subsequent within-group, three-way, repeated-measures ANOVA revealed an intervention \times day \times time interaction in the HIB group only ($P=0.042$). In the HIB group, TNF- α increased by 29% after EVH at day 0, and this response was unchanged after placebo. Conversely, after B-GOS, baseline TNF- α was reduced (mean difference = 0.50 (SD 0.61)pg/ml; 95% CI 0.02, 0.96pg/ml, $P=0.04$) and the 29% increase in TNF- α after EVH was completely abolished ($P=0.002$) (Fig. 4).

Serum chemokines, C-reactive protein and IgE

In control and HIB groups, serum CCL11, CCL17, CRP and IgE were unchanged after every EVH test; therefore, subsequent analyses were performed on baseline data only. In the control group, baseline CCL11 and CCL17 were unchanged after both interventions. In the HIB group, baseline CCL11 was unchanged after placebo and B-GOS, and CCL17 was unchanged after placebo. Conversely, *a priori* Wilcoxon's signed-rank tests revealed a reduction in baseline CCL17 after B-GOS ($P=0.005$; effect size = -0.88) (Fig. 5).

In control and HIB groups, baseline CRP was unchanged after placebo, whereas *a priori* Wilcoxon's signed-rank tests revealed a reduction in CRP in control ($P=0.050$; effect size = -0.49) and HIB ($P=0.015$; effect size = -0.57) groups

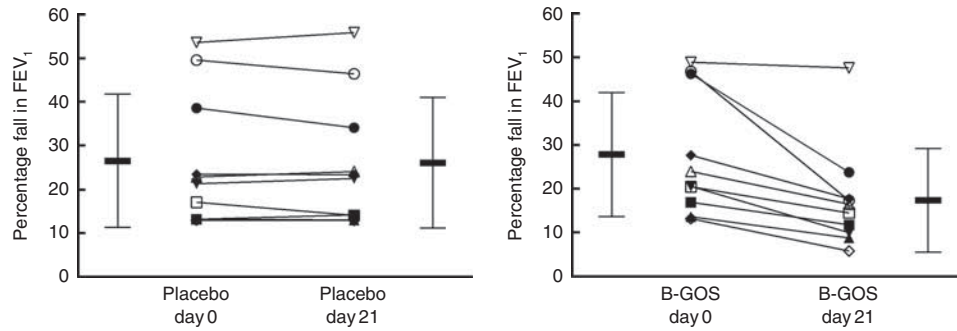


Fig. 3. The individual data and mean values and standard deviations for the peak fall in forced expiratory volume in 1 s (FEV_1) after eucapnic voluntary hyperpnoea in hyperpnoea-induced bronchoconstriction participants only. Individual participants are represented by the same symbols in both the placebo and B-GOS figures. B-GOS, Bimuno-galactooligosaccharides.

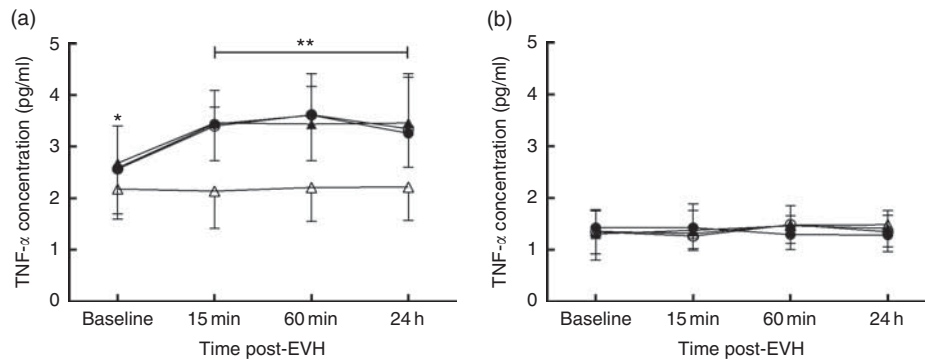


Fig. 4. $TNF-\alpha$ concentration at baseline and after eucapnic voluntary hyperpnoea (EVH) in hyperpnoea-induced bronchoconstriction (a) and control (b) groups. Values are means and standard deviations. Post-EVH values were averaged for statistical analysis. Significant difference: B-GOS day 0 v. B-GOS day 21 (* $P=0.04$; ** $P=0.002$). ●, Placebo day 0; ○, placebo day 21; ▲, Bimuno-galactooligosaccharides (B-GOS) day 0; △, B-GOS day 21.

after B-GOS (Fig. 5). Baseline IgE was higher in the HIB group (pooled data: 37 (SD 17) KU/l) than in the control group (pooled data: 14 (SD 8) KU/l) ($P=0.006$) and no changes were observed after placebo or B-GOS.

Fraction of exhaled nitric oxide

In the HIB group, baseline F_{ENO} (pooled data: 45 (SD 30) ppb) was unchanged after placebo and B-GOS.

Discussion

The main finding of this study was that supplementation with B-GOS in adults with asthma attenuated the fall in pulmonary function after EVH (reduced peak falls in FEV_1 and PEF) and the overall severity of bronchoconstriction ($\Delta FEV_1 AUC_{0-30}$). Furthermore, B-GOS reduced baseline concentrations of CCL17, CRP and $TNF-\alpha$ and abolished the EVH-induced increase in $TNF-\alpha$. These findings suggest that B-GOS can potentially mediate the underlying immunopathology of asthma, and thereby attenuate the airway hyper-responsiveness associated with HIB/EIB.

The 40% (370 ml) improvement in the post-EVH fall in FEV_1 after B-GOS supplementation exceeds the minimum perceptible change of 230 ml⁽²⁶⁾, and is therefore clinically relevant.

Prophylactic use of β_2 -agonists for EIB prevention/protection is common because of their efficacy. For example, salbutamol (200 μ g) and salmeterol (50 μ g) delivered before exercise reduced the fall in FEV_1 by approximately 78 and 61%, respectively^(27,28). Comparatively β_2 -agonists therefore offer greater protection than B-GOS; however, they are not curative, and some individuals with EIB either do not respond to β_2 -agonists or experience a reduction in severity but not symptoms⁽²⁹⁾. Furthermore, chronic use of β_2 -agonists causes tolerance or β_2 -receptor desensitisation with associated symptom exacerbation⁽²⁹⁾. B-GOS is well tolerated^(12,14), and the mechanisms of action may modify the underlying immunopathological features of asthma^(16,30).

The mechanisms by which prebiotics ameliorate airway hyper-responsiveness remain unclear. Previous studies report that B-GOS robustly supports the growth of bifidobacteria in the human gut^(11–15) and these microbes may interact with the intestinal mucosal immune system to enhance immunomodulatory effects. Dendritic cell sampling of bifidobacteria and lactobacilli may alter naïve T-cell differentiation by promoting an increase in regulatory T-cells expressing Forkhead box protein P3 (Foxp3) and, subsequently, increased production of anti-inflammatory cytokines IL-10 and TGF- β ⁽³¹⁾. Suppression of effector T-cell formation may partially explain the observed changes in $TNF-\alpha$, which is released by mast cells, neutrophils, eosinophils and airway epithelial cells and causes airway

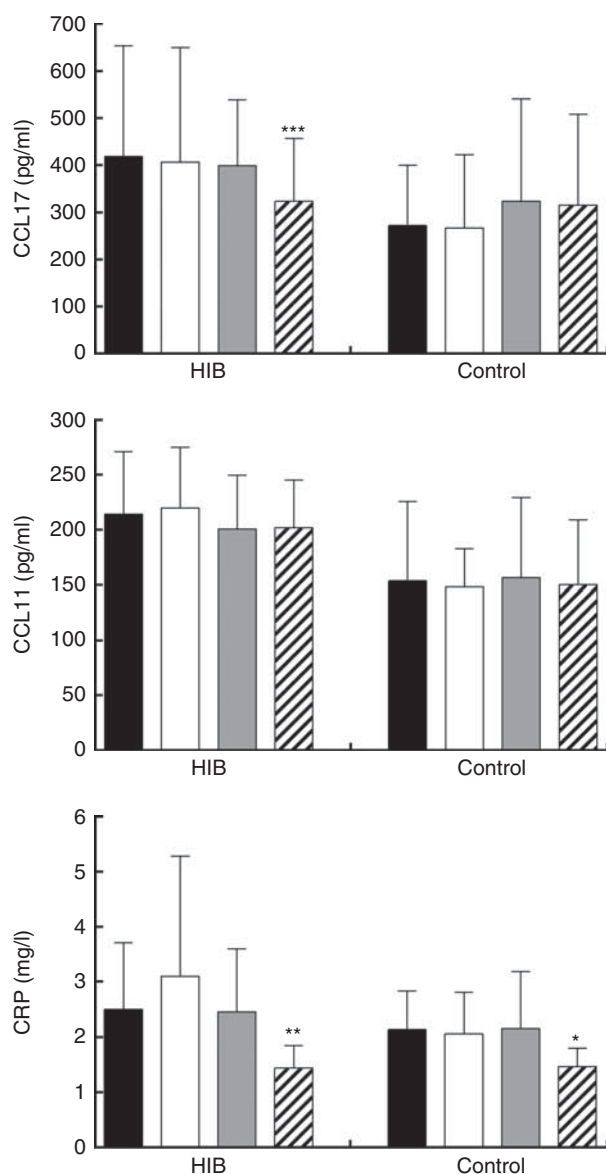


Fig. 5. Baseline concentrations of the chemokine CC ligand 17 (CCL17) and chemokine CC ligand 11 (CCL11) and C-reactive protein (CRP). Values are means and standard deviations represented by vertical bars. Significant difference: Bimuno-galactooligosaccharides (B-GOS) day 0 v. B-GOS day 21 (* $P=0.05$; ** $P=0.015$; *** $P=0.005$). ■, Placebo day 0; □, placebo day 21; ■, B-GOS day 0; ▨, B-GOS day 21.

hyper-responsiveness. The HIB group had a higher baseline TNF- α concentration than the control group, which concurs with previous reports of raised TNF- α in athletes with EIB⁽³²⁾. In the HIB group, B-GOS supplementation reduced baseline TNF- α and, remarkably, abolished the EVH-induced increase in TNF- α . Interestingly, reduced baseline TNF- α concentration after B-GOS supplementation was previously observed in the elderly⁽¹²⁾. Consequently, the reduced severity of HIB observed after B-GOS supplementation may be explained by modulation of the immune system by the gut microbiota, which results in an attenuated inflammatory response to increased osmolarity of the airway surface liquid during/following EVH.

Allergic asthma and EIB are characterised by increased T_H2 cell infiltration, which is partly controlled by the release of CCL11, CCL17 and CCL5 from airway epithelium and bronchial smooth muscle cells^(33,34). Baseline serum CCL17 concentration was similar to that reported previously in individuals with allergic asthma⁽³⁵⁾ and tended to be higher in the HIB group than in the control group. The reduction in CCL17 following B-GOS supplementation in the HIB group further indicates reduced systemic chemokine expression, which may lower infiltration of T_H2 lymphocytes into the airways.

Serum CRP is associated with airway inflammation, obstruction and bronchial hyper-responsiveness in individuals with asthma⁽³⁶⁾. Consistent with previous reports in the elderly⁽¹²⁾, we observed a reduction in CRP in both HIB and control groups after B-GOS. Conversely, F_{ENO} and serum IgE were unchanged after B-GOS. F_{ENO} is a marker of eosinophilic airway inflammation that is raised in individuals with asthma⁽¹⁷⁾. However, because of the heterogeneity of the F_{ENO} and IgE measures in the current cohort, a detectable reduction may require a longer duration of B-GOS supplementation.

Faecal samples were not collected in the present study to verify that B-GOS increased the numbers of bifidobacteria. However, we are confident that this was the case, as the prebiotic index of B-GOS, which measures the increase in the number of beneficial bacteria (*Bifidobacterium* and *Lactobacillus-Enterococcus*) compared with the reductions in less-favourable bacteria (*Bacteroides-Prevotella* and *Clostridium perfringens-histolyticum*), was 0.40 (SD 0.13)⁽¹¹⁾, and previous studies have consistently reported an increase in the number of bifidobacteria within the gut after B-GOS supplementation in humans^(11–15).

In conclusion, B-GOS supplementation reduced the severity of HIB, and this was associated with reduced systemic concentrations of T_H2-driven inflammatory markers. These findings suggest that B-GOS, through its impact on the gut microbiota, has the potential to modulate the underlying immunopathology of asthma, and thereby attenuate the airway hyper-responsiveness associated with HIB/EIB. The precise mechanisms by which B-GOS modulates immune function and reduces airway inflammation remain unclear and warrant further exploration.

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N. C. W., M. A. J., D. E. S., G. R. S. and K. A. H. designed the study; N. C. W., M. A. J., G. R. S. and K. A. H. conducted the study; N. C. W., J. V. and I. S. provided essential reagents and conducted analysis of blood samples; N. C. W., M. A. J. and G. R. S. analysed the data; N. C. W., M. A. J., G. R. S. and K. A. H. wrote the paper; N. C. W., M. A. J., D. E. S., I. S., J. V., G. R. S. and K. A. H. contributed to reviewing and approval of the final manuscript.

J. V. is employed by Clasado Research Services who supplied the supplement but were not involved in the design of the study. None of the other authors had personal or financial conflicts of interest.

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