

Butyrylated starch increases colonic butyrate concentration but has limited effects on immunity in healthy physically active individuals.

Nicholas P West, Claus T Christophersen, David B Pyne, Allan W Cripps, Michael A Conlon, David L Topping, Seungha Kang, Chris S McSweeney, Peter A Fricker, Daniel Aguirre, Julie M Clarke

Physiology, Australian Institute of Sport (NPW, DBP, PAF), School of Medicine, Griffith University (NPW, AWC), Commonwealth Scientific and Industrial Research Organization (CSIRO) Animal, Food and Health Sciences (CTC, MC, DLT, JMC), Livestock Industries, (SK, CSM, DA, MM).

Abstract

Background: Butyrate delivery to the large bowel may positively modulate commensal microbiota and enhance immunity.

Objective: To determine the effects of increasing large bowel butyrate concentration through ingestion of butyrylated high amylose maize starch (HAMSB) on faecal biochemistry and microbiota, and markers of immunity in healthy active individuals.

Design: Male and female volunteers were assigned randomly to consume either two doses of 20 g HAMSB ($n=23$; age 37.9 ± 7.8 y; mean \pm SD) or a low amylose maize starch (LAMS) ($n=18$; age 36.9 ± 9.5 y) twice daily for 28 days. Samples were collected on days 0, 10 and 28 for assessment of faecal bacterial groups, faecal biochemistry, serum cytokines and salivary antimicrobial proteins.

Results: HAMSB led to relative increases in faecal free (45%; 12-86%; mean; 90% confidence interval; $P=0.02$), bound (950%; 563-1564%; $P<0.01$) and total butyrate (260%; 174-373%; $P<0.01$) and faecal propionate (41%; 12-77%; $P=0.02$) from day 0 to day 28 compared to LAMS. HAMSB was also associated with a relative 1.6-fold (1.2- to 2.0-fold; $P<0.01$) and 2.5-fold (1.4- to 4.4-fold; $P=0.01$) increase in plasma IL-10 and TNF- α but did not alter other indices of immunity. There were relative greater increases in faecal *P. distasonis* (81-fold (28- to 237-fold; $P<0.01$) and *F. prausnitzii* (5.1-fold (2.1- to 12-fold; $P<0.01$) in the HAMSB group.

Correspondence and requests for reprints:
Nicholas West, Griffith University, PMB 50,
Gold Coast Mail Centre Queensland, Australia, 9726
Email: n.west@griffith.edu.au

Conclusions: *HAMSB supplementation in healthy active individuals promotes the growth of bacteria that may improve bowel health and has only limited effects on plasma cytokines.*

Key words: Butyrylated starch, immunity, exercise, short chain fatty acids, faecal microbiota

Introduction

Prolonged physical and psychological stress as experienced by elite athletes (12, 39), nurses (24) and students undertaking examinations (25), can increase susceptibility to illness and disease via perturbations in immune function. Stress can lower the salivary concentrations of immunoglobulin A (IgA), lactoferrin and lysozyme, and other humoral factors of the immune system that provide defence at mucosal surfaces. Lower salivary IgA is correlated with an increased risk of upper respiratory tract illness (URTI) (14) and a recent study also showed that dys-regulated cytokine responses to an exercise challenge test were associated with a higher rate of URTI in active individuals (8). There may be potential to support the immune system through diet and lifestyle changes which would reduce the risk of stress-induced illnesses.

Altering the abundance of beneficial colonic microbiota and increasing the concentration of bacterial-derived metabolites, in particular short-chain fatty acids (SCFAs), has the potential to improve gut health and immune function. A substantial body of research strongly indicates that commensal bacteria play a key role in health and disease through interaction with epithelial and immune cells (26, 29). The colonic microbiota is also responsible for the fermentation of undigested dietary carbohydrates and the production of SCFAs, principally acetate, propionate and butyrate. These SCFAs play a key role in the function of epithelial cells and the mucosal barrier in the colon (5). Butyrate in particular has attracted considerable attention as a major metabolic fuel for colonocytes and has been reported to have a wide range of effects on immune cell functions including chemotaxis, phagocytosis, reactive oxygen species production, cytokine/chemokine release and immune cell migration (2, 36, 37). Butyrate's effects may occur via the activation of G protein coupled receptors (GPR) which are expressed on immune cells, adipocytes and intestinal epithelial cells. GPR41 and 43 have the highest affinity for SCFAs (33) and are both expressed by cells in the colonic epithelium and GPR43 is also highly expressed in circulating immune cells.

Bacterial fermentation of fibre increases the abundance of commensal bacteria and increases the concentration of SCFAs. Resistant starch (RS) is a component of dietary fibre, which, when fermented in the large bowel favors the production of butyrate and increases the abundance of the commensal bacteria *P. distasonis* (7, 9, 28, 35). The ingestion of acylated starches has the capacity to deliver specific SCFAs to the colon (6). Acylated starches in which SCFAs are esterified to a carrier starch to a moderate degree of substitution (DS: the average number of

hydroxyl groups on each D-glucopyranosyl unit derivatized by substituent groups (34) resist small intestinal digestion and pass to the colon where the esterified acids are liberated by bacterial enzymes. The residual starch is fermented with the production of further SCFAs. Butyrylated starch has proven effective at delivering butyrate to the large bowel of healthy humans and increasing colonic SCFA (7). This was, however, in a specific population cohort with low butyrate concentrations and further research in populations with normal faecal butyrate concentrations is necessary.

The aim of this study was to investigate the effects of increasing colonic butyrate through the ingestion of butyrylated starch on gut health and immunity in healthy, active individuals. To achieve this aim a 28 day parallel design, double blind, randomized, controlled trial was undertaken and markers of gut, mucosal and systemic immunity were measured to determine the potential of butyrylated starch to improve the health of physically active individuals.

Methods

Subjects

Following screening 41 healthy active male ($n=23$; age 37.9 ± 7.8 y, mean \pm SD) and female ($n=18$; age 36.9 ± 9.5 y) cyclists were enrolled to provide 80% statistical power and an alpha of 0.05 on the primary outcome measures based on the literature and the authors' experience. These individuals were recruited from the general cycling community in Canberra Australia by email and personal contact at cycling events. Subjects were required to declare their use of dietary and/or performance enhancing substances that may influence underlying immune function. All subjects consuming immuno-modulatory medications or the regular use of any drug, medication or supplement which could interfere with bowel function were excluded. Inclusion to the study was dependent upon the subjects not taking antibiotics and supplements or foods with probiotics for at least one month prior to and during the study period. The study statistician allocated the participants to treatment groups using minimisation stratified by sex. Participants and the study team were blinded as to the allocation of treatments. All procedures involving human subjects were approved by the Human Research Ethics Committees of the Australian Institute of Sport (AIS), CSIRO and Griffith University. All participants provided written informed consent. The study was conducted according to the guidelines prescribed in the Declaration of Helsinki and registered with the Australian New Zealand Clinical Trials Registry ACTRN12611000771954.

Study design

The study was a randomized, double blind, parallel controlled trial designed to compare the effects of ingestion of 40g/day of butyrylated high amylose maize starch (HAMSB) with low amylose maize starch (LAMS; control). The study consisted of a 14-day pre-intervention period where subjects were asked to refrain from eating yoghurt and any supplements that could modulate enteric microbiota. This baseline phase was followed by a 28-day period when subjects consumed the supplements. At the end of the baseline period (day 0) and at day 14 and day 28 of the supplementation period subjects provided samples for the assessment of fae-

cal microbiology and biochemistry, and immune function. Subjects were paired on age and maximal oxygen uptake ($\text{VO}_{2\text{max}}$), and randomly allocated to either experimental (HAMSB) or placebo supplementation by the study statistician who did not have direct contact with the subjects.

During supplementation subjects consumed two specially formulated beverages containing 20g of the test starches daily; one in the morning and one in the evening. All subjects maintained a four-day food diary in the second week of the study that incorporated two weekdays and a weekend to determine total daily energy (kJ) and fibre (g) intakes. Subjects were also required to maintain two two-day food diaries prior to providing the faecal samples on days 0 and 28. These records were used to identify any short term changes in starch consumption which could modify faecal microbiota and SCFA. Dietary analysis was undertaken using FoodWorks professional edition software package (version 3.0, Xyris Software, Brisbane, Australia). At the end of the study subjects completed a gastrointestinal quality of life questionnaire (GIQLI) to examine the effects of supplementation on GI function as described previously (7).

Test Product

The specially formulated beverages comprised 60 g of Protein Plus Protein Powder[®] (Powerbar Oceania, Rhodes, Australia) with either 20 g HAMSB (Ingredient Incorporated, formerly National Starch and Chemical Company, Bridgewater, NJ) or LAMS (New Zealand Starch, Auckland, New Zealand) in 200 ml of milk or water. HAMSB had a DS of 0.23 as determined by CSIRO Materials Science and Engineering by use of ¹³C-NMR spectroscopy using a Bruker BioSpin DRX500 NMR spectrometer (Fällanden, Switzerland). Subjects were asked to consume one beverage in the morning and one in the evening. The beverages were consumed with or without food.

Sample collection

Saliva, blood and faecal samples were obtained pre- (day 0), mid- (day 14) and end-of supplementation (day 28) from all subjects. Saliva was collected with an oral eyespear swab (Defries Industries Pty Ltd, Victoria, Australia) for determination of IgA, lactoferrin and lysozyme. The eyespear was placed between the cheek and teeth for 5 min, removed and centrifuged immediately for 5 min at 778 g and then frozen at -80°C until analysis. Albumin concentration was assessed to control for changes in salivary flow rate. All saliva samples were taken at the same time of the day to control for diurnal variation. A blood sample (9 ml) was drawn from the antecubital vein to quantify resting serum cytokine concentrations. Each sample was collected directly into a K₃EDTA tube (Greiner Bio-one; Frickenhausen, Germany) and frozen at -80°C until analysis. Participants provided a faecal sample within 48 h of the blood and saliva sampling. Faeces were collected in a sealable plastic bag and frozen immediately at -20°C in a portable freezer until transfer to laboratory storage at -80°C.

Measures of mucosal immunity

Lactoferrin, lysozyme and SIgA concentrations were measured spectrophotometrically by enzyme linked immunosorbant assay (ELISA) using commercial kits

(lactoferrin – EMD Chemicals, New Jersey, USA; lysozyme – Sapphire Bioscience, Redfern, Australia; SIgA – Salimetrics, IgA – Salimetrics, Philadelphia, USA). Albumin concentration was measured by immunoturbidimetric assay on a Hitachi 911 Chemistry Analyzer (Roche). Osmolality was measured on a Model 3320 Osmometer (Advanced Instruments Inc) as per the manufacturer's instructions. Variability was acceptable at <10% for the low and high positive controls.

Plasma cytokines

Granulocyte macrophage-colony stimulating factor, (GM-CSF), interleukin (IL)-1RA, IL-6, IL-8, IL-10, tumour necrosis factor (TNF)- α and interferon gamma (IFN- γ) cytokines were measured on a Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). The plasma samples were analyzed on custom manufactured Multiplex Cytokine Kits (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). Plates were read using the Bio-Plex Suspension Array System (Bio-Rad Laboratories Pty Ltd; Hercules, CA, USA). A full blood count including white cell count and differential was performed on a haematology analyser (Advia, GMI, Michigan, USA). Results from each assay were accepted if the positive controls were within two standard deviations of their established mean concentration. Each plate included a control.

Illness, training and performance measures

Symptoms of gastrointestinal illness were recorded daily by subjects as described previously (11). Details of exercise or physical training undertaken during the study were also recorded. For each session, training mileage ($\text{km}\cdot\text{wk}^{-1}$), duration ($\text{h}\cdot\text{wk}^{-1}$) and intensity (scored on a 1–5 scale: 1, easy; 5, maximal) were recorded. From this data a value for weekly training load can be ascertained by multiplying training duration in hours by intensity. At the start of the study subjects undertook an incremental exercise test to exhaustion to determine peak power output and maximal oxygen uptake (VO_2max). The test was performed on an electromagnetic cycle ergometer (Excalibur Sport, Lode NV Groningen, Netherlands) as described previously (20).

Microbial analysis

DNA extraction

DNA was extracted from faecal samples as previously described (41). Briefly, the protocol combines a mechanical (bead beating) and enzymatic lysis of bacterial cells followed by a cleanup to eliminate contamination from other cell debris.

Phylogenetic profile using a custom microarray

A custom phylogenetic microarray developed and validated for human gut bacteria was used to analyze the microbiota (17). Briefly, extracted DNA from faecal samples from days 0 and 28 were amplified using the prokaryote 16S rRNA gene primer sets 27F (5'– AGAGTTTGATCMTGGCTCAG–3') and T7/1492R (5'– TCTAATACGACTCACTATAGGGGGYTACCTTGTTACGACTT–3') (the underlined region is modified to include a T7 promoter sequence). Detailed methods of cRNA synthesis and labeling, hybridization, image capture and analysis have been previously described (17).

Quantitative real time PCR (Q-PCR)

Q-PCR was performed on samples collected at days 0, 14 and 28 to confirm the findings of the microbial changes identified with the human gut microarray. Total bacteria, *Faecalibacterium prausnitzii* and *Parabacteroides distasonis* were quantified using Q-PCR, which were performed in reaction volumes of 10 or 20 μ l (*P. distasonis*) containing 1X SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA) and 0.2 mg/ml BSA. Primers (concentration): Total bacteria (150 nM), 1114f (5'-CGGCAACGAGCGCAACCC-3') and 1275r (5'-CCATTGTAGCACGTGTGTAGCC-3') (10); *F. prausnitzii* (500 nM), FPR-1 (5'-AGATGGCCTCGCGTCCGA-3') and FPR-2 (5'-CCGAAGACCTTCTTCCTCC-3') (16), and *P. distasonis* (500 nM) BdisF (5'-TGATCCCCTGTGCTGCT-3') and BdisR (5'-ATCCCCCTCATTCGGA-3') (21). For quantification a total of 10 ng of template DNA was used and Q-PCR cycling was performed in a Chromo-4 thermocycler (Bio-Rad Laboratories, Hercules, CA). The Q-PCR cycling conditions were as follows: 4 min at 98°C followed by 35 cycles of 98°C for 20 s, 60-62°C for 20 s (total bacteria: 60°C; *F. prausnitzii* and *P. distasonis*: 62°C) and 72°C for 30 s with fluorescent acquisition after each cycle. A final meltcurve analysis was performed after completion of all cycles with fluorescence acquired at 0.5 °C intervals between 55 and 95°C to verify that only the expected fragment was amplified. Q-PCR product was also visualized on a 1.5% agarose gel. Non-template controls were included and assays were performed in triplicate by analyzing the same DNA sample in 3 independent reactions. An 8-series of 10-fold dilutions of a sample derived plasmid construct (Top chemical competent cells, Invitrogen) containing the target amplicon were analyzed in parallel with DNA samples for estimation of absolute abundance and Q-PCR efficiency for all assays. Results were analyzed with the Opticon Monitor 3 software (ver. 3.1) (Bio-Rad Laboratories, Hercules, CA). All Q-PCR data were analyzed as absolute numbers of bacteria in one gram of wet weight faeces.

Short chain fatty acids

Faecal samples were thawed at 4°C and then subsampled for analysis. Weighed portions for the determination of free (unesterified) SCFA were diluted 1:3 w/w with deionized water containing 1.68 mM heptanoic acid as an internal standard (Sigma Chemical Co, St Louis, MO). Unesterified SCFA were analyzed as described previously (4). A three point linear standard curve containing acetic, propionic, isobutyric, butyric, isovaleric, valeric and caproic acids was used for calibration at concentrations spanning the range of those measured in samples from this study. Total SCFA concentrations were determined as described previously (6) with the exception that hydrolysis was undertaken by agitating the samples for 2 h with 0.7 times the sample volume of 6 M NaOH. A subset of seven samples with a wide range of butyrate concentrations were analyzed twice using both methods to ensure consistency and accuracy of the revised method. Total faecal ammonia concentration and pH were determined using previously described methods (3).

Statistical analysis

All data is presented as mean \pm standard deviation. Filtered and normalized microarray data were analyzed by the multivariate analysis tool, principal compo-

nant analysis (PCA) using the Genespring program, which determined automatically the number of components in the PCA models. Volcano plot analysis was also performed on the data with more than two-fold differences (> 2 fold) and significant P-value (P=0.05) between different diets (HAMSB and LAMS) and time points (days 0 and 28) in Genespring 7.3 software (Agilent Technologies, Santa

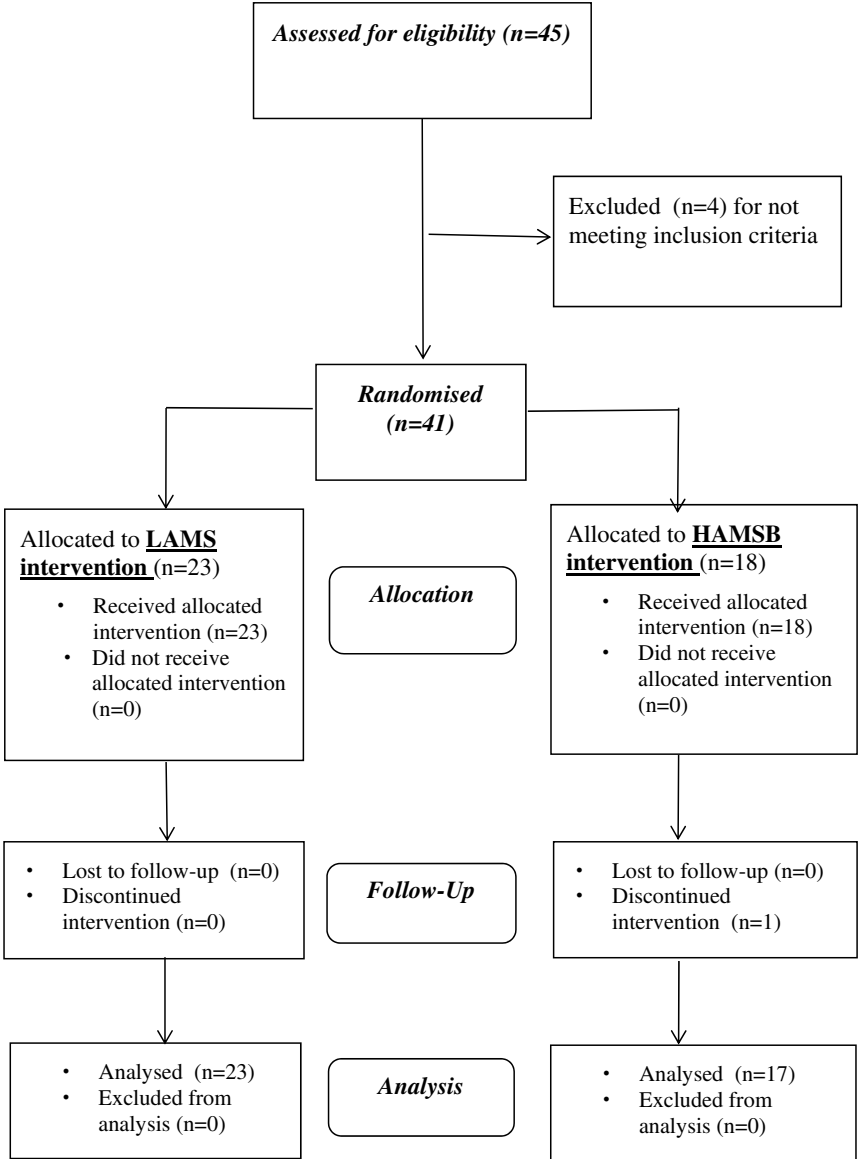


Figure 1. Flow of participants through the study using a Consort Flow Diagram.

Clara, CA, USA). The selected probes filtered by volcano plot analysis were compared by box plot using the same software and differences were confirmed by Q-PCR analysis. Statistical analysis of measures of immunology and enteric microbiota (Q-PCR data) evaluated the magnitude of the difference in the mean change between the treatment groups from day 0 to days 14 (mid-supplementation) and 28 (end of supplementation).

The measures of physical activity, salivary and serum proteins, faecal biochemistry and Q-PCR microbiological data were all log-transformed before analysis to reduce non-uniformity of error and permit the effect of the treatment to be analyzed as a percent. Differences in the change in mean saliva and serum protein concentrations, and Q-PCR faecal data between the groups were analyzed with a Student's t-test for independent samples (unequal variance). Baseline values of the dependent variable were included as a covariate in these analyses to account for regression to the mean. The extent to which changes in bacterial counts accounted for changes in other outcome measures was examined through covariate analysis.

Standardized mean changes were used to characterize differences between groups. A modification of Cohen's effect size (ES) classification system (trivial: 0.0–0.2; small: 0.2–0.6; moderate: 0.6–1.2; large: 1.2–2.0) was used to interpret the magnitude of observed changes (15). The effects of supplementation are shown with 90% confidence limits. Statistical significance was accepted at $P < 0.05$.

Results

Subjects

A total of 45 individuals volunteered and were assessed for eligibility. The flow of participants through the study and the consort checklist is shown in Figure 1. Four individuals who did not meet the inclusion criteria were excluded. Of the 41 individuals recruited one male participant in the HAMS group withdrew from the study within seven days due to the taste of the supplement.

There were no significant differences in baseline characteristics between the groups at allocation (Table 1). Three female and two male volunteers reduced their intake of supplementation from 40 g of supplement to 20 g in the first seven days due to a feeling of fullness. Data were analyzed from all subjects allocated to a treatment group. Dietary analysis exclusive of the supplements found no significant differences between the groups in intakes of total energy (kJ) (HAMS 8954 ± 2870 versus LAMS 8069 ± 1850) or fibre (g) (HAMS 19 ± 12 versus LAMS 16 ± 7) during the study. HAMS supplementation was not associated with a change in symptoms of GI discomfort as measured by the GIQLI (data not shown). Examination of the two 2-day food diaries showed no instances of volunteers consuming foods high in RS immediately before the faecal collection days of the study. There were no significant differences between the groups in the number of exercise training days per week (HAMS 4.4 ± 2.0; LAMS 4.5 ± 2.1) or training load (HAMS 4.26 ± 1.22; LAMS 4.23 ± 1.73).

Table 1. Baseline characteristics of study volunteers

	LAMS	HAMSB	P-value
n	23	18	
Age (y)	37.2 ± 8.5	37.6 ± 8.3	0.87
Mass (kg)	69.0 ± 14.2	66.0 ± 11.2	0.45
VO ₂ max (ml.min ⁻¹ .kg ⁻¹)	57.5 ± 5.0	54.9 ± 7.2	0.18

Data are mean ± SD. VO₂max, maximal oxygen uptake.

Faecal measures

Changes and differences in selected faecal measures between HAMSB and LAMS are shown in Table 2. The change in faecal pH from day 0 to day 14 was lower in the HAMSB group 14 (HAMSB -0.11 ± 0.37 versus LAMS 0.22 ± 0.49 , mean ± SD, $P=0.02$) but not from day 0 to day 28 (HAMSB 0.03 ± 0.29 versus LAMS 0.21 ± 0.45 , $P=0.13$). There was a substantial reduction in the concentration of ammonia in the first 14 days in the HAMSB group (-33%; -12 to -50%, mean; ±90% confidence interval) but not LAMS (17%; 0 - 36%, $P=0.01$). The difference between the groups in the mean change in faecal ammonia concentration from pre- to post-supplementation was not significant.

Changes in the concentration of total SCFA, acetate, propionate, butyrate are also presented in Table 2. There was a relative 38% (10 - 74%; $P=0.01$) difference in total SCFA concentration between the groups from pre- to mid-study, with HAMSB increasing total SCFA concentration by 14% (-4 to 36%) and LAMS reducing total SCFA concentration by 19% (-32 to -54%). No significant effects were evident in total SCFA concentration between the groups from pre- to post-supplementation. There was a moderate difference between the groups in the concentration of acetate (28%; 2 - 60%; $P=0.07$) from pre- to mid-supplement (mean change, HAMSB -1.3%; ±18% versus LAMS -21%; ±20%, mean; ±90% confidence limits) but no substantial difference between the groups in acetate concentration after 28 days. The concentration of propionate increased by 25% (7 - 47%) in the HAMSB group with no substantial change in the LAMS group from pre- to post-supplementation. The ratio of acetate to propionate was reduced by 30% (20-38%; $P<0.01$) in the HAMSB group compared to the LAMS group over the course of the study. Supplementation with HAMSB over 28 days yielded significantly higher increases of ~45% in free butyrate, ~10-fold in bound butyrate, and ~2.5 fold in total butyrate compared with LAMS (Table 2).

Covariate analysis did not reveal any clear trends between changes in *P. distasonis* and *F. prausnitzii* and changes in SCFA. Furthermore, no clear relationship between changes in SCFA, individually or in total, was evident with changes in faecal pH or ammonia.

Table 2. The effect of supplementation for 14 and 28 days with LAMS and HAMS on faecal biomarkers.

Parameter	LAMS				HAMS				Difference in Change (Day 14 - 0)		Difference in Change (Day 28 - 0)	
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28	Day 0	Day 14	Mean %; Mean \pm 99%CL	P-value	Mean %; Mean \pm 99%CL	P-value
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD				
Faecal output (g)	155 \pm 94	189 \pm 98	135 \pm 95	136 \pm 54	127 \pm 61	183 \pm 78	136 \pm 54	127 \pm 61	-29; \pm 33	0.05	65; \pm 42	0.02
Ammonia $\mu\text{mol.g}^{-1}$	22 \pm 13	22 \pm 9	18 \pm 8	23 \pm 13	16 \pm 9	17 \pm 7	23 \pm 13	16 \pm 9	-36; \pm 37	0.02	-5; \pm 25	0.55
Total SCFA $\mu\text{mol.g}^{-1}$	83 \pm 21	71 \pm 30	78 \pm 40	86 \pm 33	99 \pm 37	84 \pm 27	86 \pm 33	99 \pm 37	45; \pm 27	0.01	-14; \pm 27	0.38
Acetate $\mu\text{mol.g}^{-1}$	52 \pm 15	44 \pm 19	50 \pm 23	54 \pm 20	54 \pm 19	47 \pm 15	54 \pm 20	54 \pm 19	28; \pm 26	0.08	-0.4; \pm 27	0.90
Propionate $\mu\text{mol.g}^{-1}$	15 \pm 4	14 \pm 6	15 \pm 7	16 \pm 7	23 \pm 9	20 \pm 6	16 \pm 7	23 \pm 9	67; \pm 28	<0.01	41; \pm 26	0.02
Free butyrate $\mu\text{mol.g}^{-1}$	16 \pm 7	13 \pm 6	13 \pm 7	16 \pm 7	23 \pm 11	18 \pm 6	16 \pm 7	23 \pm 11	77; \pm 35	<0.01	46; \pm 29	0.02
Bound butyrate $\mu\text{mol.g}^{-1}$	6 \pm 5	5 \pm 4	6 \pm 7	6 \pm 3	58 \pm 32	47 \pm 29	6 \pm 3	58 \pm 32	618; \pm 102	<0.01	950; \pm 58	<0.01
Total butyrate $\mu\text{mol.g}^{-1}$	21 \pm 7	18 \pm 8	20 \pm 11	21 \pm 7	81 \pm 41	65 \pm 31	21 \pm 7	81 \pm 41	192; \pm 50	<0.01	260; \pm 31	<0.01

CL – confidence limit; g – gram; μmol – micromole; SD – standard deviation

Faecal microbiology

Microarray analysis comparing microbial profiles between day 0 and day 28 showed clear differences between the responses to HAMS and LAMS treatments. Principal components analysis (PCA) showed differences in the microbial profiles after treatments of LAMS samples compared to HAMS (Figure 2). Microarray analysis revealed post-HAMS treatment group had a higher *P. distasonis* signal than other groups. Similarly samples from some individuals

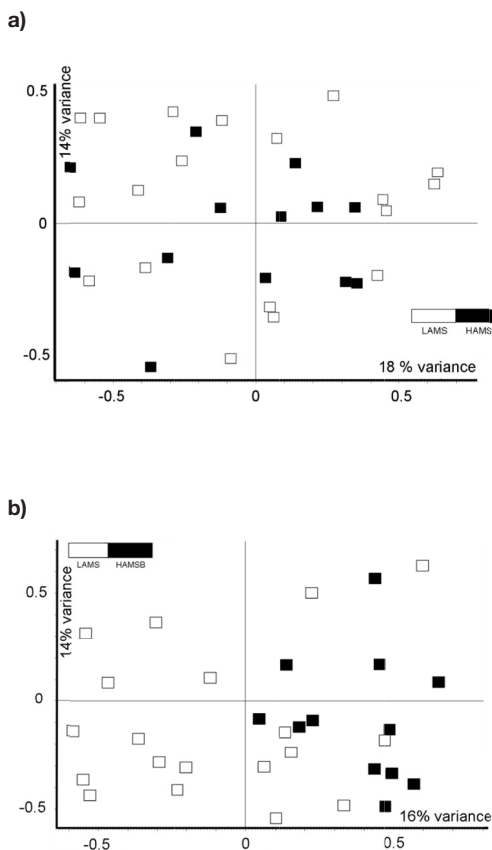


Figure 2. Principal component analysis plots showing the % variation of faecal microbial diversity explained by the x-axis and y-axis. Plot A) shows faecal microbial diversity between HAMSB and LAMS on day 0. Plot B) shows a substantial change in microbial diversity between HAMSB and LAMS on day 28. Individual data points represents a sample with black squares representing HAMSB samples and clear squares represents LAMS samples.

confidence interval). From day 0 to day 28 there was a greater decline in IL-10 in the LAMS group than the HAMSB group (by a factor of 1.6-fold; 1.2- to 2.0-fold; $P < 0.01$) while the change in TNF- α concentration was 2.5-fold (1.4- to 4.4-fold; $P = 0.01$) higher in the HAMSB group compared with the LAMS group. Starch supplementation did not affect changes in serum IL-6, IL-8 or GM-CSF concentrations. There were no significant effects of supplementation on other plasma cytokines (GM-CSF, IL-6, IL-8, and IFN- γ).

showed a significant increase in signal intensity of the *F. prausnitzii* probes in response to HAMSB but the mean difference was not significant for the whole treatment group (data not shown).

These differences were confirmed by Q-PCR using primers specific for *P. distasonis* and *F. prausnitzii* (Figure 3). HAMSB supplementation elicited an 81-fold increase in *P. distasonis* and a 5.1-fold increase in the abundance of *F. prausnitzii* from day 0 to day 28 compared to LAMS. Differences in total bacteria between the treatment groups were not significant.

Plasma cytokines

The effect of supplementation on resting plasma concentrations of IL-1RA, IL-10 and TNF- α is shown in Figure 4. IL-1RA concentration was 1.9-fold higher in the HAMSB group compared with the LAMS group from day 0 to day 14. However, there was only a trivial difference between the groups from 0 to day 28 of supplementation. The differences between the groups from day 0 to day 14 in IL-1RA were a result of a reduction in IL-1RA concentration in the LAMS group (-43%; -55 to -28; 90% confidence interval).

Mucosal immunity

There were no substantial differences between the treatment groups over the course of the study in any of the salivary proteins measured: compared with LAMS the HAMS group had a 10% (-19% to 50%; $P=0.61$) increase in the concentration of SIgA, a 7% (-43% to 95%; $P=0.86$) increase in salivary lysozyme and a 3% (-35% to 30; $P=0.80$) decrease in the concentration of salivary lactoferrin from day 0 to day 28.

Discussion

This study confirms the findings of previous studies that HAMS supplementation raises faecal butyrate concentrations (7), which is consistent with the documented ability of acylated starches to deliver SCFAs to the human large bowel (6). The study demonstrated also that supplementation with HAMS substantially increased the faecal numbers of *P. distasonis* and maintained those of *F. prausnitzii*. Similar increases in the faecal numbers of *P. distasonis* have been reported in humans (7) and animals (1) consuming HAMS. In contrast, consumption of LAMS substantially lowered the faecal numbers of *P. distasonis* and *F. prausnitzii* and also the concentrations of propionate and butyrate. Collectively these data suggest HAMS may promote large bowel health in healthy active individuals and that diets containing refined starch (such as LAMS) may have detrimental effects on colonic health. Walker et al (38) reported that substitution of a highly digestible starch for foods containing significant quantities of resistant starch raised

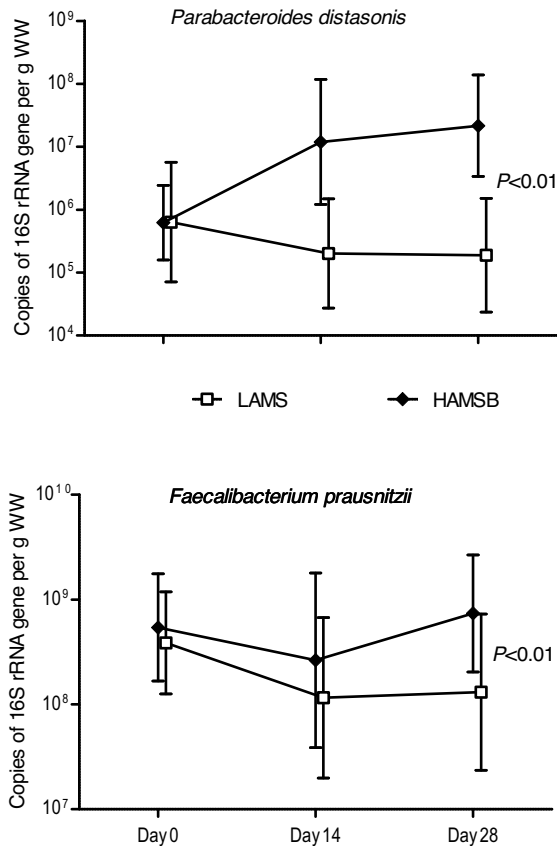


Figure 3. The effect of supplementation with 40 g.day⁻¹ HAMS and LAMS on fecal *P. distasonis* and *F. prausnitzii* (mean ± SD). *significant difference between the treatment groups from day 0 to day 28.

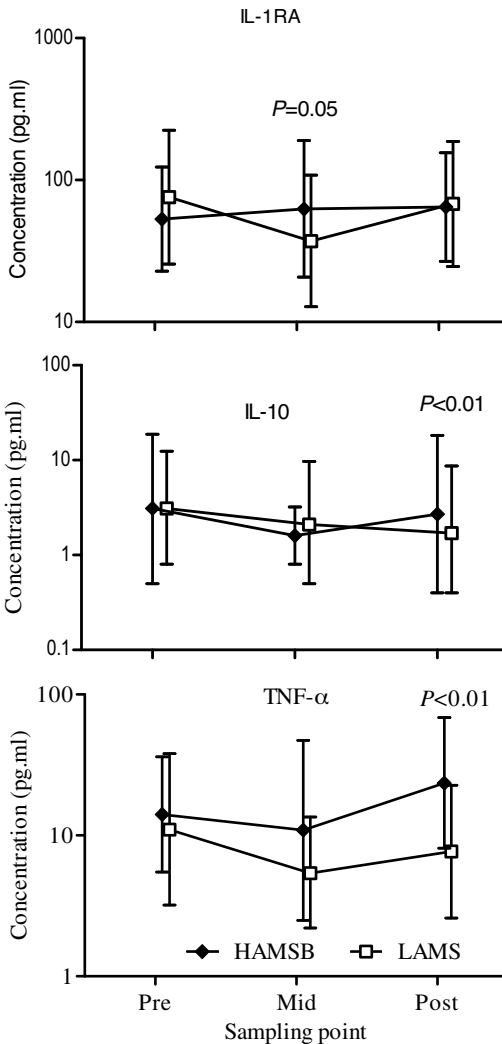


Figure 4. The effect of supplementation on the concentration of IL-1RA, IL-10 and TNF- α from pre- to post-intervention (mean \pm SD). *significant difference ($P < 0.01$) between the treatment groups over the course of the study.

study. Furthermore, there were moderate decreases in the concentration of butyrate in the LAMS group. The explanation for the reduction in butyrate concentration in the HAMSB group from day 14 to day 28 is uncertain given there were no substantial changes in dietary practices and compliance throughout the study, although the increase in faecal bulk in this last 14 days suggests ongoing adaptation in the large bowel. The role of butyrate as a fuel for colonocytes and

faecal pH in African school-children supporting the latter suggestion.

Previous animal experiments have shown that acylated starches raise large bowel SCFA after the release of the esterified SCFA and subsequent fermentation of the residual starch. This combined action explains the higher faecal concentrations of all major SCFAs as well as the reduction in pH values in the HAMSB group in the present study. The latter effect is thought to occur via direct acidification of gut contents by SCFAs and, while animal results are conflicting (1) by lower NH_3 levels as well. In this study covariate analysis found no clear trend between the increasing SCFA and lower NH_3 levels with pH.

The ileal digestibility of LAMS is $\sim 100\%$ and we anticipated its consumption would not alter any of the faecal biomarkers which we measured (6). However, this was not entirely the case as HAMSB and LAMS had divergent effects on faecal propionate, butyrate and pH. As expected, butyrate concentrations (bound, free and total) substantially increased with HAMSB supplementation but increases in free butyrate were only evident to day 14 of the

in epithelial integrity is well recognized (32) and this study provides support for increased uptake and utilization with prolonged ingestion. The question as to whether the lower butyrate concentration at day 28 reflects increased utilization needs to be the focus of future research. The reason for the difference in total SCFA concentration between the groups to the mid-point of the study relates to both a fall in total SCFA concentration in the LAMS group and a substantial increase in the HAMS group.

The substantial falls in butyrate and total SCFA concentration and the increases in faecal pH and ammonia in the LAMS group may be related to the replacement of fermentable carbohydrates with LAMS during the study. The continued refinement of starches in modern food manufacturing has resulted in an increasing consumption of simple starches. The findings from this study indicate that these starches may reduce the abundance of beneficial bacteria and concentration of short chain fatty acids.

Commensal microbiota plays an important role in gut and immune development and homeostasis and the prevention of disease (22). Estimates for faecal abundance of *P. distasonis* and *F. prausnitzii* showed no differences between the two groups at entry into the trial. The dose of the HAMS supplement ($40 \text{ g} \cdot \text{person}^{-1} \cdot \text{day}^{-1}$) was sufficient to alter faecal biomarkers, promote an increase in faecal recovery of *P. distasonis*, and maintain the level of faecal *F. prausnitzii*. In contrast, consumption of LAMS lowered substantially the faecal recovery of both bacterial species. *F. prausnitzii* favors butyrate production, which is of considerable importance in light of the putative role of this SCFA in bowel health. Numbers of this bacterium are low in Crohn's disease and irritable bowel syndrome patients compared to healthy controls (17, 31). However a recent report of clinical improvement in Crohn's disease correlated with a substantial decrease in *F. prausnitzii* abundance (16), highlighting the complexity in understanding the role of the microbiota in health and disease. Oral administration of *P. distasonis* reduced the severity of intestinal inflammation in a mouse model of colitis (18). This study provides evidence that HAMS promotes selective growth of potentially beneficial bacteria in healthy active individuals while consumption of LAMS reduced the number of those organisms.

There were indications of an effect of supplementation on selected circulating immune markers while mucosal (salivary) indices were unchanged. Cytokines have an essential role in regulating and coordinating immune activity. We observed a substantial difference from day 0 to day 28 in the concentration of TNF- α between the groups, due in part to a decrease in TNF- α concentration in the LAMS group from days 0 to 14. Furthermore, the concentration of the anti-inflammatory cytokine IL-10 was maintained in the HAMS group in comparison to the LAMS group, which had a moderate reduction in IL-10 from days 0 to 28. Other inflammatory markers were unchanged.

An important benefit of exercise is its anti-inflammatory effect in protecting host tissues from micro-trauma and damage to skeletal muscle (27). However, a possible consequence of this down-regulation of inflammation is an increased suscep-

tibility to common infections, particularly upper respiratory tract illness, in people undertaking prolonged intense exercise (13). Evidence indicates that anti-inflammatory cytokines may increase susceptibility to infection (13). In the context of this sample of healthy active adults and the hypothesis that exercise promotes an anti-inflammatory cytokine profile, maintaining serum TNF- α concentration with HAMS consumption may ameliorate this risk of infection. It should also be noted, however, that the fall in the circulating concentration of the anti-inflammatory cytokine IL-10 in the LAMS group may also moderate the risk of illness associated with an enhanced anti-inflammatory profile from exercise. There is evidence from colitis animal models and *in-vitro* studies of colitis that *P. distasonis* and *F. prausnitzii* modulate cytokine production from resident tissue immune cells, the gut and peripheral blood mononuclear cells (19, 30). *F. prausnitzii* has been demonstrated to induce IL-10 secretion, which may explain the maintenance of this cytokine in the HAMS group compared to the LAMS group. With the exception of TNF- α , the effect of supplementation with HAMS on cytokine concentration in this study are consistent with other research which found no substantial effects of 25 g HAMS supplementation per day for four weeks on serum cytokines (40). Serum cytokines are linked with chronic low grade inflammation that underlies the pathogenesis of many chronic developed world diseases (23). In this context determining whether butyrylation of HAMS contributed to the increase in serum TNF- α concentration and also the role of LAMS in reducing anti-inflammatory cytokines is necessary. Given the strengthening link between inflammation, metabolic dys-regulation and chronic disease, understanding the role of HAMS and LAMS may have important implications for dietary guidelines on starch consumption.

In conclusion, this study confirms the beneficial effects of supplementation with HAMS on markers of bowel health in healthy physically active adults. Furthermore, supplementation with LAMS had potentially detrimental effects on these parameters and on markers of inflammation. The potential for HAMS to support the health and performance of healthy active individuals undertaking prolonged, stressful exercise warrants further investigation.

Acknowledgements

The authors would like to thank the participants for their time and cooperation. We also acknowledge members of the Department of Physiology, Australian Institute of Sport (particularly Dr Andrew Vogler), and CSIRO Food and Nutrition Sciences for their assistance during the study. Financial support was provided by the Australian Institute of Sport and the CSIRO Preventative Health Research Flagship. All authors read and approved the final manuscript. There are no conflicts of interest to declare.

References

1. Abell G, Christophersen CT, McOrist AL, and Clarke JM. Dietary resistant and butyrylated starches have different effects on the faecal bacterial flora of azoxymethane-treated rats. *Br J Nutr* 105: 1480-1485, 2011.
2. Bailon E, Cueto-Sola M, Utrilla P, Rodriguez-Cabezas ME, Garrido-Mesa N, Zarzuelo A, Xaus J, Galvez J, and Comalada M. Butyrate in vitro immune-modulatory effects might be mediated through a proliferation-related induction of apoptosis. *Immunobiology* 215: 863-873, 2010.
3. Bajka BH, Clarke JM, Cobiac L, and Topping DL. Butyrylated starch protects colonocyte DNA against dietary protein-induced damage in rats. *Carcinogenesis* 29: 2169-2174, 2008.
4. Bajka BH, Topping DL, Cobiac L, and Clarke JM. Butyrylated starch is less susceptible to enzymic hydrolysis and increases large-bowel butyrate more than high-amylose maize starch in the rat. *Br J Nutr* 96: 276-282, 2006.
5. Cherbut C. Inulin and oligofructose in the dietary fibre concept. *Br J Nutr* 87 Suppl 2: S159-162, 2002.
6. Clarke JM, Bird AR, Topping DL, and Cobiac L. Excretion of starch and esterified short-chain fatty acids by ileostomy subjects after the ingestion of acylated starches. *Am J Clin Nutr* 86: 1146-1151, 2007.
7. Clarke JM, Topping DL, Christophersen CT, Bird AR, Lange K, Saunders I, and Cobiac L. Butyrate esterified to starch is released in the human gastrointestinal tract. *Am J Clin Nutr* 94: 1276-1283, 2011.
8. Cox AJ, Pyne DB, Saunders PU, Callister R, and Gleeson M. Cytokine responses to treadmill running in healthy and illness-prone athletes. *Med Sci Sports Exerc* 39: 1918-1926, 2007.
9. Cummings JH, Beatty ER, Kingman SM, Bingham SA, and Englyst HN. Digestion and physiological properties of resistant starch in the human large bowel. *Br J Nutr* 75: 733-747, 1996.
10. Denman SE, and McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol* 58: 572-582, 2006.
11. Fricker PA, Pyne DB, Saunders PU, Cox AJ, Gleeson M, and Telford RD. Influence of training loads on patterns of illness in elite distance runners. *Clin J Sport Med* 15: 246-252, 2005.
12. Gleeson M, and Bishop NC. Special feature for the Olympics: effects of exercise on the immune system: modification of immune responses to exercise by carbohydrate, glutamine and anti-oxidant supplements. *Immunol Cell Biol* 78: 554-561, 2000.
13. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, and Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat Rev Immunol* 11: 607-615, 2011.
14. Gleeson M, McDonald WA, Pyne DB, Cripps AW, Francis JL, Fricker PA, and Clancy RL. Salivary IgA levels and infection risk in elite swimmers. *Med Sci Sports Exerc* 31: 67-73, 1999.
15. Hopkins WG, Marshall SW, Batterham AM, and Hanin J. Progressive statistics for studies in sports medicine and exercise science. *Med Sci Sports Exerc* 41: 3-13, 2009.

16. Jia W, Whitehead RN, Griffiths L, Dawson C, Waring RH, Ramsden DB, Hunter JO, and Cole JA. Is the abundance of *Faecalibacterium prausnitzii* relevant to Crohn's disease? *FEMS Microbiol Lett* 310: 138-144, 2010.
17. Kang S, Denman SE, Morrison M, Yu Z, Dore J, Leclerc M, and McSweeney CS. Dysbiosis of fecal microbiota in Crohn's disease patients as revealed by a custom phylogenetic microarray. *Inflamm Bowel Dis* 16: 2034-2042, 2010.
18. Kverka M, Zakostelska Z, Klimesova K, Sokol D, Hudcovic T, Hrnecir T, Rossmann P, Mrazek J, Kopecny J, Verdu EF, and Tlaskalova-Hogenova H. Oral administration of *Parabacteroides distasonis* antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. *Clin Exp Immunol* 2010.
19. Kverka M, Zakostelska Z, Klimesova K, Sokol D, Hudcovic T, Hrnecir T, Rossmann P, Mrazek J, Kopecny J, Verdu EF, and Tlaskalova-Hogenova H. Oral administration of *Parabacteroides distasonis* antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. *Clin Exp Immunol* 163: 250-259, 2011.
20. Lee H, Martin D, Anson J, Grundy D, and Hahn A. Physiological characteristics of successful mountain bikers and professional road cyclists. *J Sports Sci* 20: 1001-1008, 2002.
21. Liu C, Song Y, McTeague M, Vu AW, Wexler H, and Finegold SM. Rapid identification of the species of the *Bacteroides fragilis* group by multiplex PCR assays using group- and species-specific primers. *FEMS Microbiol Lett* 222: 9-16, 2003.
22. Macdonald TT. Inside the microbial and immune labyrinth: totally gutted. *Nat Med* 16: 1194-1195, 2010.
23. Mathur N, and Pedersen BK. Exercise as a mean to control low-grade systemic inflammation. *Mediators Inflamm* 2008: 109502, 2008.
24. Ng V, Koh D, Chan G, Ong HY, Chia SE, and Ong CN. Are salivary immunoglobulin A and lysozyme biomarkers of stress among nurses? *J Occup Environ Med* 41: 920-927, 1999.
25. Ng V, Koh D, Mok B, Lim LP, Yang Y, and Chia SE. Stressful life events of dental students and salivary immunoglobulin A. *Int J Immunopathol Pharmacol* 17: 49-56, 2004.
26. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, and Pettersson S. Host-gut microbiota metabolic interactions. *Science* 336: 1262-1267, 2012.
27. Pedersen BK, Steensberg A, Keller P, Keller C, Fischer C, Hiscock N, van Hall G, Plomgaard P, and Febbraio MA. Muscle-derived interleukin-6: lipolytic, anti-inflammatory and immune regulatory effects. *Pflugers Arch* 446: 9-16, 2003.
28. Phillips J, Muir JG, Birkett A, Lu ZX, Jones GP, O'Dea K, and Young GP. Effect of resistant starch on fecal bulk and fermentation-dependent events in humans. *Am J Clin Nutr* 62: 121-130, 1995.
29. Smith PM, and Garrett WS. The gut microbiota and mucosal T cells. *Front Microbiol* 2: 111, 2011.
30. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, Blugeon S, Bridonneau C, Furet JP, Corthier G, Grangette C, Vasquez N, Pochart P, Trugnan G, Thomas G, Blottiere HM, Dore J, Marteau P, Seksik P, and Langella P. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* 105: 16731-16736, 2008.

31. Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, Cosnes J, Corthier G, Marteau P, and Dore J. Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. *Inflamm Bowel Dis* 15: 1183-1189, 2009.
32. Tarini J, and Wolever TM. The fermentable fibre inulin increases postprandial serum short-chain fatty acids and reduces free-fatty acids and ghrelin in healthy subjects. *Appl Physiol Nutr Metab* 35: 9-16, 2010.
33. Tazoe H, Otomo Y, Karaki S, Kato I, Fukami Y, Terasaki M, and Kuwahara A. Expression of short-chain fatty acid receptor GPR41 in the human colon. *Biomed Res* 30: 149-156, 2009.
34. Thomas D, and Atwell W. *Starches: Practical guides for the food industry* (p. 94). Saint Paul, Minnesota: Eagan Press, 1999.
35. van Munster IP, Tangerman A, and Nagengast FM. Effect of resistant starch on colonic fermentation, bile acid metabolism, and mucosal proliferation. *Dig Dis Sci* 39: 834-842, 1994.
36. Vinolo MA, Rodrigues HG, Hatanaka E, Hebeda CB, Farsky SH, and Curi R. Short-chain fatty acids stimulate the migration of neutrophils to inflammatory sites. *Clin Sci (Lond)* 117: 331-338, 2009.
37. Vinolo MA, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, and Curi R. Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J Nutr Biochem* 22: 849-855, 2011.
38. Walker AR, Walker BF, and Segal I. Faecal pH value and its modification by dietary means in South African black and white schoolchildren. *S Afr Med J* 55: 495-498, 1979.
39. West NP, Pyne DB, Kyd JM, Renshaw GM, Fricker PA, and Cripps AW. The effect of exercise on innate mucosal immunity. *Br J Sports Med* 44: 227-231, 2010.
40. Worthley DL, Le Leu RK, Whitehall VL, Conlon M, Christophersen C, Belobrajdic D, Mallitt KA, Hu Y, Irahara N, Ogino S, Leggett BA, and Young GP. A human, double-blind, placebo-controlled, crossover trial of prebiotic, probiotic, and synbiotic supplementation: effects on luminal, inflammatory, epigenetic, and epithelial biomarkers of colorectal cancer. *Am J Clin Nutr* 90: 578-586, 2009.
41. Yu Z, and Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* 36: 808-812, 2004.