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Resistant potato starches (type 4 RS) exhibit varying effects on laxation with and without phylum level changes in microbiota: A randomised trial in young adults

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ABSTRACT

The effects of resistant potato starches on gastrointestinal (GI) function and microbiota in healthy individuals were investigated. In a 6-week, double-blind, cross-over study, subjects (N = 57; 21M; 36F) were randomised to consume 30 g fibre/d from one of three chemically modified potato starches (RS4-A, soluble, viscous; RS4-B, soluble, non-viscous; RS4-C, insoluble, non-viscous) and control in beverages for 2 weeks with a 1-week washout and daily reporting of stool frequency, Bristol Stool Form Scale (BSFS), GI symptoms and compliance. Faecal microbiota was analysed by qPCR and 16S rRNA sequencing. Stool frequency and BSFS increased only with RS4-B ($P < 0.01$). GI symptoms were minimal with slight increases in flatulence with all interventions ($P < 0.001$). There were no changes in *Lactobacillus* or *Bifidobacteria* spp. However, RS4-B decreased Firmicutes ($P = 0.02$) and the ratio of Firmicutes to Bacteroidetes ($P = 0.01$). Resistant potato starches vary in their effects on GI function, which may be related to shifts in intestinal microbiota.

1. Introduction

The current average intake of fibre in North America remains far below recommendations (Reicks, Jonnalagadda, Albertson, & Joshi, 2014); thus an increased use of functional fibre ingredients may be required to improve total fibre intakes. Resistant starches (RS), including type 1 RS (physically inaccessible), type 2 RS (compact crystalline structure), type 3 (retrograded), type 4 RS (chemically modified) and type 5 RS (amylose-lipid complexes), contribute to the fibre content of foods (Raigond, Ezekiel, & Raigond, 2015). Resistant starches (RS) are well suited for fibre fortification given their ease of addition to food products and generally acceptable functional and sensory characteristics (Raigond et al., 2015). RS has been shown to increase faecal weight but not stool frequency (Baer et al., 2014; Maki et al.,

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2009), and to have little effect on GI symptoms (Fastinger et al., 2008; Klosterbuer et al., 2013; Lefranc-Millot et al., 2012; Martinez, Kim, Duffy, Schlegel, & Walter, 2010; Stewart, Nikhanj, Timm, Thomas, & Slavin, 2010; Storey, Lee, Bornet, & Brouns, 2007). Studies suggest that at least 20 to 25 g/d of RS may be required to achieve significant changes in laxation (Maki et al., 2009).

The health benefits of RS may extend beyond the potential benefits of laxation. It is well known that naturally occurring RS provides substrate for short chain fatty acid (SCFA) production (Bird, Conlon, Christophersen, & Topping, 2010), and there is mounting evidence supporting the positive effects of SCFA production on appetite and energy homeostasis (Byrne, Chambers, Morrison, & Frost, 2015), as well as mitigation of inflammation (Kim, Park, & Kim, 2014). In addition, the therapeutic benefit of improving insulin resistance with type 2 RS has been demonstrated in type 2 diabetes (Karimi et al., 2015) and metabolic syndrome (Johnston, Thomas, Bell, Frost, & Robertson, 2010).

Fermentative activity of the gut microbiota, such as the quantity and the ratio of the major short chain fatty acids (SCFA) produced, i.e. acetate to butyrate to propionate, may be modulated by RS to confer these health-enhancing effects. Animal studies support that fermentation of RS contributes beneficial effects to the gut (e.g. modulation of gene expression, proliferation and apoptosis) and systematic effects on adiposity and insulin resistance (Keenan et al., 2015). *In vitro* models suggest that type 3 RS, a resistant maltodextrin, increases production of butyrate (Brouns et al., 2007). In pigs, a retrograded tapioca starch, a type 3 RS, was shown to increase caecum and faecal SCFA, primarily acetate and propionate, with an increase in butyrate seen only in the colon (Haenen et al., 2013). In rats, pregelatinised, cross-linked and acetylated type 4 RS was shown to increase caecum SCFA, primarily propionate, whereas only the starch cross-linked with sodium trimetaphosphate increased butyrate levels in the caecum (Le Thanh-Blicharz et al., 2014). As *in vivo* measurements of SCFA production in proximal human colon are challenging due to difficulty accessing the luminal contents, utilisation of SCFA by the colonic epithelium (primarily butyrate), and lack of access to the portal blood supply to determine absorption (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987), human studies must rely on *in vitro* methods and faecal sampling. High amylose starches have been shown to increase butyrate levels in an *in vitro* fermentation model (Li et al., 2015). In a recent study in obese men, a commercially available type 3 RS resulted in a decreased faecal concentrations of acetate, propionate, butyrate and succinate compared to a maintenance diet; however, total faecal output was not determined precluding conclusions related to total faecal content of SCFA (Salonen et al., 2014).

While changes in microbiota occur throughout the gastrointestinal tract, faeces represent a convenient sample for analysing effects of RS on its composition. Colonic fermentation of RS is thought to modify human gut microbiota composition and activities, specifically increasing potentially beneficial bacteria and butyrate production, and decreasing opportunistic pathogens, suggesting a prebiotic effect (Bird et al., 2010; Zaman & Sarbini, 2015). The potential effects of RS on microbiota may be dependent on the type of RS. Most research to date has evaluated type 3 RS, retrograded starches, with less research exploring the effects of type 4 RS, chemically modified starches. Lefranc-Millot et al. demonstrated that 8 to 20 g of resistant dextrin, a type 3 RS, increased *Bacteroides* spp. and inhibited *Clostridium perfringens* (Lefranc-Millot et al., 2012). However, Fastinger et al. evaluated the effects of two doses of a resistant maltodextrin (7.5 and 15 g/d) on gastrointestinal function and symptoms, and microbiota, and found no significant changes in *Bifidobacterium* genus, *Lactobacillus* genus or *Clostridium perfringens* (Fastinger et al., 2008). Klosterbuer et al. compared soluble maize fibre to a type 3 RS at 20–25 g/d fibre and saw no significant changes in microbiota (Klosterbuer et al., 2013). In obese men on controlled diets, a type 3 RS diet increased *Ruminococcaceae* phylotypes, but decreased the overall diversity of the microbiota (Salonen et al., 2014). In Malawi children, type 2 RS resulted in phylum level changes, with an increase in Actinobacteria and a decrease in Firmicutes, as well as increases in *Lactobacillus* and decreases in certain genera such as *Roseburia* (Ordiz et al., 2015). With regard to type 4 RS, Martinez et al. (2010) examined the effect of 33 g/d of phosphorylated cross-linked type 4 RS in 10 subjects and found a phylum level change in microbiota, specifically a decrease in Firmicutes and an increase in Bacteroidetes and Actinobacteria, as well as genus level changes including an increase in *Bifidobacteria*. Paturi et al. (2012) tested a type 4 RS in rats and demonstrated an increase in *Lactobacillus* and *Bifidobacteria* spp. compared to diet with cellulose. *In vitro* studies provide supporting data that there is the potential for chemically modified starch to enhance the growth of *Bifidobacteria* and *Lactobacillus* spp. (Slizewska, 2013).

Further research is needed to explore the effects of type 4 RS on the intestinal microbiota, but also on their physiological efficacy as functional fibres. The objectives of this study were to determine the effects of three chemically modified resistant potato starches on stool frequency, stool form, gastrointestinal symptoms and faecal microbiota in healthy individuals.

2. Materials and methods

2.1. *Subjects*

Staff and students at the University of Florida, Gainesville, FL, USA (18–65 y), were recruited via flyers, posters and announcements. Potential study volunteers were excluded if they had a history of gastrointestinal disease or food allergy, consumed prebiotic or fibre supplements, or had a usual fibre intake of >20 g/d. This study was approved by the Institutional Review Board at the University of Florida and conducted according to guidelines established by the Declaration of Helsinki. Subjects provided written consent indicating their full knowledge of the study protocol.

2.2. *Experimental design*

A 6-week randomised, double-blinded crossover intervention study was carried out during the spring of 2014. Based on a previous studies in this same population and with stool fre-

quency as the primary outcome (Dahl et al., 2014), at $\alpha = 0.05$ and power of 0.80, 17 to 21 subjects per group were needed. Baseline 24-h diet recalls using the Automated Self-Administered 24-hour Recall (ASA24) system (NIH, 2012) were completed for 7 days to determine usual energy and fibre intake. Height was determined, using a wall-mounted Stadiometer (Seca, Hanover, MD, USA) and weight in kilogram using a Health o Meter electronic scale (Sunbeam Products Inc, Boca Raton, FL, USA). Demographic information was obtained at baseline. Subjects were randomised (by sealed envelopes containing subject assignments prepared by a research associate who did not have contact with the subjects) to one of three resistant modified potato starches providing 30 g of fibre from RS4-A (PenFibe® RO – 170; phosphorylated, soluble fibre with high viscosity), RS4-B (PenFibe® RO – 177; hydrolysed, phosphorylated, soluble fibre with low viscosity), or RS4-C (PenFibe® RS; insoluble fibre with low viscosity) (Penford Food Ingredients Inc., Denver, CO, USA) and control in fruit-flavoured beverages (Kool-Aid®, Kraft Foods Inc., Northfield, IL, USA). Two beverages a day, each containing 15 g/serving of fibre (in addition to the subjects' background diet fibre intake), were provided for a two-week period separated by a one-week washout. The Kool-Aid® vehicle provided 168 kcal/day.

The primary outcome measure assessed in this study was stool frequency. Secondary outcome measures included stool form and gastrointestinal symptoms. Throughout the study, subjects completed online daily questionnaires reporting stool frequency, stool form using the Bristol Stool Form Scale (BSFS), gastrointestinal symptoms (bloating, flatulence, abdominal cramping, abdominal noises) rated as follows: 1–no discomfort, 2–slight discomfort, 3–mild discomfort, 4–moderate discomfort, 5–moderately severe discomfort, 6–severe discomfort and 7–very severe discomfort, as well as study beverage compliance. The study coordinators monitored subject compliance on a daily basis.

2.3. *Microbiota analysis*

An additional secondary outcome measure assessed in this study was modulation of microbiota.

From each subject, a total of six stool samples (one baseline, two treatment, two control and one washout) were collected for DNA isolation for microbiota studies. Stools were collected using a commode specimen collection system (Fisher Scientific, Pittsburgh, PA, USA). Participants were instructed to place the collection systems containing the samples on ice immediately after defecation and to deliver samples to study personnel within four hours of defecation. Samples were weighed and homogenised by kneading. Samples were then aliquoted and stored at –80 °F until analysis. DNA from the faecal samples was extracted using a QIAamp DNA Stool Kit (Qiagen, Hilden, Germany) and included a bead beating step. The qPCR reactions were carried out in duplicate with an initial melting step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 sec, 58 °C for 60 sec and 72 °C for 1 min. Primer sequences were as follows: for *Bifidobacteria*, Bif forward 5'-GATTCTGGCTCAGGATGAACG-3'; Bif reverse 5'-CGGGTGCTICCCCACTTTCATG-3'; and for lactic acid bacteria (LAB), LAB forward 5'-ACGAGTAGGGAATCTTCCA-3' (Kaufmann, Pfefferkorn, Teuber, & Meile, 1997); LAB reverse 5'-ATTYCACCGCTACACATG-3' (Walter et al., 2001). All reactions were performed using 10 ng of DNA and 0.2 μmol/L of each primer and difference calculated per ng of DNA.

The16S rRNA gene (V1–V2 Region) was amplified using validated primers (Koren et al., 2011): MiSeq 27F 5'-AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT CC AGMGTTYGATYMTGGCTCAG-3' containing 5' Illumina adapter; primer pad; primer linker; and Forward primer. MiSeq-338R (reverse primer) PCR primer sequence (each sequence contains different barcode) was used with the sequence 5'- CAAGCAGAAGACGGCATACGAGAT TCCCTTGTCTCC AGTCAGTCAG AA GCTGCCTCCCGTAGGAGT-3' containing reverse complement of 3' Illumina adapter; Golay barcode; primer pad; primer linker; and reverse primer. The PCR conditions were initial melting step at 95 °C for 2 min, followed by 20 cycles of 95 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1 min 30 sec. Two 50 μl PCR reactions for each sample were combined together and the PCR products were purified with Agencourt AMPure XP system (Beckman Coulter). Cleaned PCR products were pooled in equimolar amounts and submitted for sequencing. Illumina MiSeq (2×250 bp) sequencing was performed using bar-coded primers with 50% PhiX spike. Four samples for each subject were sequenced, baseline, end of treatment period, washout, and end of control period. After removal of low quality reads, those with short read length or with low quality score, a total of 6,705,083 sequences were retained, a mean of 28,680 sequences per sample with an average length of 319 nucleotides. A modified UPARSE algorithm was used, in a dedicated microbiome analysis pipeline (Pylro et al., 2014), for binning sequences into Operational Taxonomic Units (OTUs) using similarity levels of 95 and 98% (BioProject ID: PRJNA309319). After removal of OTUs containing less than 10 sequences, 1615 and 2937 OTUs were retained at the respective similarity level.

2.4. *Statistical analyses*

For daily stool frequency (SF), daily average Bristol Stool Form Scale (a-BSFS), and individual stool BSFS form converted to three categorical variables (c-BSFS) (BSFS ≤ 2 = slow transit; BSFS 3 to 5 = normal transit; BSFS > 5 = fast transit), a generalised linear mixed model with fixed effects of period (1 or 2), phase (baseline or intervention), treatment (pairs: control or RS4-A, control or RS4-B, control or RS4-C) and all two- and three-way interactions were fitted. A random effect of subject was included to account for daily repeated observations. For SF, the fitted distribution was quasi-Poisson, with variance equal to the mean times an underdispersion parameter. The natural log link was used. For a-BSFS, the fitted distribution was normal. For the c-BSFS fitted distribution was multinomial with a cumulative logit link, and if the phase × treatment interaction was significant, two category variables were created (BSFS ≤ 2 = slow transit, BSFS > 2 = normal transit; BSFS > 5 = fast transit, ≤ 5 normal transit). The fitted distributions were binary with a logit link.

Gastrointestinal symptom scores were analysed using a generalised linear mixed model with fixed effects as described above. For bloating, flatulence, cramping and abdominal noise, although the data are integer scores ranging from 1 to 7, the fitted distributions were approximately normal. The

bloating variable was found to be skewed with unequal variances, and was therefore natural-log transformed before analysis. For cramping and noise, a model was fitted to a 2-category variable created from cramping (no cramping/noise < 2; cramping/noise >1). The fitted distributions for the category variables were binary with a logit link.

For all models residuals were checked and the Kenward-Roger method was used to correct for the bias in estimates of the variances and covariances due to small sample size. For multiple comparisons of means, the Tukey-Kramer method was used to control the family-wise error rate at $\alpha = 0.05$. The covariance of the repeated observations was tested for statistical significance using a Wald test and a χ^2 test. For all variables, this covariance was found to be significant. In addition to the F-tests of the model's fixed effects, the differences in means between the two phases (baseline and intervention) within each combination of treatment and period, between the two treatments within each combination of phase and period, between the two treatments within a phase, between phases within each treatment, and between the two treatments within each period were compared.

Data for qPCR was log transformed, and two-tailed t-tests were used to test for significance between repeated samples and baseline/washout periods and respective intervention periods. A one-way ANOVA was conducted to test for significance between the interventions, and a Mann–Whitney was conducted to control for non-normalised data. Unless stated otherwise, data represent the mean ± SEM with significance denoted at $P < 0.05$.

The QIIME package was used to calculate (i) Chao rarefaction diversity, which estimates how many OTUs are present in a sample, and (ii) UniFrac distances, which allow for a comparison of the distribution of OTUs among samples. Shannon diversity indices were calculated for each sample using the total number of OTUs per sample and relative volume of each OTU using the formula $H = -\sum_{i=1}^{N} p_i \ln p_i$. For analysing differences in the prevalence of OTUs, a z score was obtained using a χ^2 test. For generating heat maps, the most significantly different OTUs were chosen based upon Z score comparing baseline of treatment to week 2 of treatment. Two-tailed t-tests were used to confirm the significance of the number of sequences. To calculate the significance of taxa proportions, two-tailed t-tests were used to test the significance between the groups for each fibre and a Mann–Whitney was conducted to control for non-normalised data.

3. Results

Following recruitment, 133 potential subjects were screened, 89 were consented, with 30 being excluded due to not meeting the post-consent inclusion criteria, no longer interested or non-compliance to baseline data collection, leaving 59 subjects to be randomised. Of these, two subjects were withdrawn due to non-compliance. The subject flow diagram is shown in Fig. 1. Table 1 gives the characteristics for subjects randomised to RS4-A, RS4-B and RS4-C. Of the 30 g/d of RS provided to subjects, compliance indicated intakes of 27 g/d, 29 g/d and 29 g/d for RS4-A, RS4-B and RS4-C, respectively. No adverse events occurred during the trial.

3.1. *Gastrointestinal function and symptoms*

In Table 2, SF and BSFS scores are given. There were no significant effects of RS4-A on SF or c-BSFS, i.e. slow, normal or fast transit. a-BSFS increased for those subjects who received the RS4-A first (Period 2), while those in Period 1, who received the control first, did not show an increase in a-BSFS with the RS4-A intervention. The gastrointestinal symptom scores are shown in Table 3. Flatulence score increased with the RS4-A, although these subjects reported lower initial scores during the baseline corresponding to the RS4-A intervention. No treatment effect was found for bloating and abdominal cramping. The probability of reporting any abdominal noise (1 vs >1) increased with RS4-A.

The RS4-B intervention significantly increased SF, a-BSFS and c-BSFS (Table 2). In addition, RS4-B showed a decrease in slow transit (BSFS < 3) and an increase in fast transit (BSFS >5) when BSFS was analysed as two categories (data not shown). Flatulence and abdominal noise increased with RS4-B, as did the probability of any reported abdominal cramping when analysed using two categories (1 vs >1) (data not shown). RS4-B had no effect on bloating (Table 3).

A trend was observed for an increase in SF with RS4-C ($P = 0.07$) (Table 2). There were no significant effects on c-BSFS and only a phase effect on a-BSFS. Flatulence and bloating increased with RS4-C, while phase effects were found for cramping and abdominal noises (Table 3).

3.2. *Microbiota*

There was no effect on total LAB and *Bifidobacteria* spp. based on targeted qPCR (data not shown) or high throughput sequencing. In addition, no difference in overall diversity based on Shannon Diversity Index was detected. At the phylum level, 16S rRNA sequence microbiota analysis showed no significant effects of RS4-A and RS4-C, suggesting that the consumption of neither of these two type 4 RS modulated the major bacterial phyla present in the healthy subjects (Fig. 2). The supplementation of RS4-B significantly decreased the proportion of Firmicutes ($P = 0.02$) with a trend for increased Bacteroidetes ($P = 0.12$), and a significant decrease in the ratio of Firmicutes to Bacteroidetes ($P = 0.01$). At the family level, *Lachnospiraceae* spp. decreased with RS4-B treatment ($P = 0.026$), primarily due to a decrease in *Coprococcus* ($P = 0.024$) (data not shown). The largest number of individual OTUs that were observed were affected by the supplementation of RS4-B; in all three groups the OTUs that decreased in prevalence upon fibre supplementation outnumbered those that increased (Fig. 3). While two OTUs matching closest to *Ruminococci* and *Faecalibacteria* increased during RS4-B supplementation, similar OTUs were either unchanged or decreased.

4. Discussion

Given the high level of supplementation, each of the resistant potato starches fed in this study might be expected to

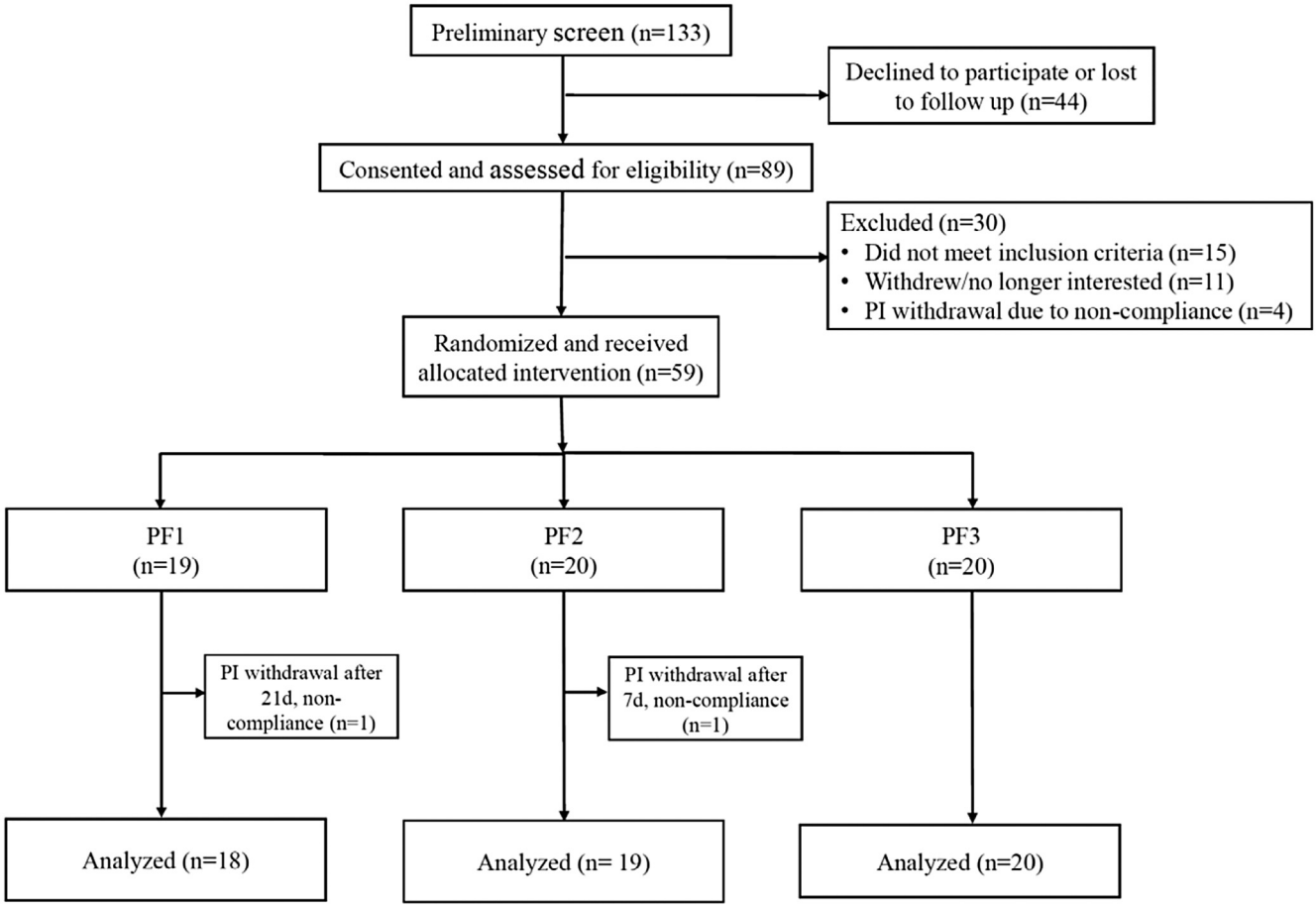

Fig. 1 – Participant flow diagram.

impact laxation. The results of this study demonstrated that only RS4-B had significant effects on both stool frequency and stool form, an indicator of gut transit time. This finding challenges the premise that stool frequency reaches a plateau at about 1 to 3 stools/d and is unaffected by further increases in fibre intake (Maki et al., 2009). In addition, the increased stool frequency is in contrast to findings with type 3 RS that did not demonstrate these changes (Baer et al., 2014; Maki et al., 2009). Symptom and microbiota data provide evidence for the fermentation of RS4-B. SCFA production and its impact on smooth muscle contractility and colonic motility may provide a mechanism by which RS4-B exerted its effects on stool frequency (Macfarlane & Macfarlane, 2011). It is possible that fermentation of RS4-B occurs slowly throughout the colon in comparison to, for example, resistant oligosaccharides (Bonnema, Kolberg, Thomas, & Slavin, 2010), thus producing low levels of symptoms and perhaps more distal effects on motility. Previous studies have shown that type 3 RS increases faecal weight (Baer et al., 2014; Maki et al., 2009). A limitation of the present study is that only single stool samples were collected, and thus daily faecal weights were not determined. It is possible that the resistant

Table 1 – Characteristics of participants randomised to three resistant potato starches, RS4-A, RS4-B and RS4-C, and their baseline energy and fibre intake.

	RS4-A	RS4-B	RS4-C
N	18	19	20
Gender, *M/F*	7M/11F	6M/13F	9M/11F
Age (y)	20.3 ± 1.7[a]	21.3 ± 2.7	20.8 ± 2.0
BMI	23.2 ± 3.7	25.3 ± 5.9	26.1 ± 4.9
Baseline daily energy intake, *kcal*	2019 ± 679	1685 ± 554	2064 ± 891
Baseline daily fibre intake, *g*	14.4 ± 3.3	13.0 ± 6.2	15.0 ± 5.1
Compliance			
Intervention beverage intake servings/d	1.8 ± 0.5	1.9 ± 0.4	1.9 ± 0.5
Estimated fibre supplementation g/d	27.0	28.5	28.5

[a] Values represent the mean ± SD.

Table 2 – Daily stool frequency and Bristol Stool Form Scale of participants consuming the control or RS4-A (N = 18), RS4-B (N = 19) and RS4-C (N = 20) beverages.

		Baseline	Intervention		Baseline	Intervention	P-values[a]
SF	Control A	1.28 ± 0.12[b]	1.24 ± 0.10	RS4-A	1.12 ± 0.10	1.25 ± 0.11	T: 0.154 I: 0.344 P: 0.656
	Control B	1.23 ± 0.11	1.17 ± 0.10	RS4-B	1.21 ± 0.11[a]	1.44 ± 0.13[b]	T: 0.014 I: 0.138 P: 0.083 P × I: 0.029 I × T: 0.004
	Control C	1.30 ± 0.16	1.28 ± 0.16	RS4-C	1.26 ± 0.16	1.42 ± 0.17	T: 0.231 I: 0.173 P: 0.806 P × I: 0.066
a-BSFS	Control A	3.54 ± 0.14	3.65 ± 0.12	RS4-A	3.48 ± 0.14	3.81 ± 0.12	T: 0.535 I: 0.005 P: 0.021 P × I: 0.030 P × I × T: 0.007
	Control B	3.55 ± 0.17	3.36 ± 0.17	RS4-B	3.36 ± 0.17[a]	4.02 ± 0.16[b]	T: 0.003 I: 0.012 P: 0.434 P × I: 0.0002 I × T: < 0.0001
	Control C	3.41 ± 0.12	3.47 ± 0.11	RS4-C	3.65 ± 0.12	3.53 ± 0.10	T: 0.035 I: 0.699 P: 0.994
c-BSFS	Control A			RS4-A			T: 0.956
	Slow	16[c]	12	Slow	18	8	I: 0.582
	Normal	79	83	Normal	78	89	P: 0.955
	Fast	5	5	Fast	4	3	
	Control B[a]			RS4-B[b]			T: 0.031
	Slow	17	18	Slow	17	11	I: 0.040
	Normal	74	76	Normal	77	72	P: 0.613
							P × I: 0.002
	Fast	8	6	Fast	6	17	I × T: 0.003
	Control C			RS4-C			T: 0.092
	Slow	18	14	Slow	12	10	I: 0.605
	Normal	76	81	Normal	80	86	P: 0.684
	Fast	6	6	Fast	8	4	

[a] P-values for treatment (control or fibre) (T), phase of intervention (baseline or intervention) (I), and period (P). Means with similar letters were significantly different at $P < 0.05$.

[b] Unless stated otherwise, values represent mean ± SEM.

[c] Percentage of stools reported as slow transit (Bristol Stool Scale form of 1 and 2), normal transit (BSFS form of 3, 4 and 5), and fast transit (BSFS form of 6 and 7).

potato starches fed in this study increased faecal weight, particularly RS4-C given its insolubility.

Consumption of each of the resistant potato starches resulted in a significant increase in flatulence, indicating fermentation of each of the resistant potato starches; however, average symptoms were minimal, suggesting that high levels of type 4 RS can be incorporated into usual diets without the concern for unpleasant or adverse symptoms. This finding is in agreement with previous research confirming that high intakes of RS produce minimal gastrointestinal symptoms (Fastinger et al., 2008; Klosterbuer et al., 2013; Lefranc-Millot et al., 2012; Martinez et al., 2010; Storey et al., 2007).

A current definition of a prebiotic "is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health" (Macfarlane, Steed, & Macfarlane, 2008). Most studies examining a prebiotic effect have assessed the impact of fermentable carbohydrate on *Bifidobacteria* and *Lactobacillus* spp., thought to be health-enhancing; however, other genus and species changes also have been reported with RS consumption. The type 4 RS products tested in the present study did not increase levels of these bacterial genera. This is in agreement with Fastinger et al. where no changes in *Bifidobacteria* and *Lactobacillus* spp. were found with a resistant dextrin (Fastinger et al., 2008), and in contrast to a report that the provision of a type 2 RS resulted in an increase in *Lactobacillus ruminus* (Abell, Cooke, Bennett, Conlon, & McOrist, 2008). However, it is important to note that intakes of near 30 g/d of each of the type 4 RS did not suppress these potentially beneficial organisms.

With the provision of RS4-B, *Ruminococcus* was one of the OTUs that increased. This finding is in agreement with Abell

Table 3 – Mean daily gastrointestinal symptoms intensities of participants consuming the control or RS4-A (n = 18), RS4-B (n = 19) and RS4-C (n = 20) beverages.

		Baseline[1]	Intervention[1]		Baseline[1]	Intervention[1]	P-values[2]	
Bloating[3]	Control 1	1.49 ± 0.07	1.58 ± 0.05	RS4-A	1.48 ± 0.07	1.70 ± 0.05	T: 0.200 I: 0.0004 P: 0.044	PxI: 0.025
	Control 2	1.17 ± 0.04	1.16 ± 0.03	RS4-B	1.09 ± 0.02[a]	1.26 ± 0.04[b]	T: 0.306 I: 0.011 P: 0.306	PxI: 0.0005 IxT: 0.006
	Control 3	1.28 ± 0.05	1.29 ± 0.04	RS4-C	1.28 ± 0.05[a]	1.41 ± 0.04[b]	T: 0.076 I: 0.032 P: 0.279	IxT: 0.046
Flatulence[3]	Control 1	1.79 ± 0.08	1.76 ± 0.06	RS4-A	1.62 ± 0.07[a]	2.03 ± 0.06[b]	T: 0.281 I: 0.002 P: 0.462	IxT: 0.0001
	Control 2	1.54 ± 0.08	1.61 ± 0.06	RS4-B	1.45 ± 0.07[a]	2.10 ± 0.07[b]	T: 0.213 I: <0.0001 P: 0.886	PxI: <0.0005 IxT: <0.0001
	Control 3	1.65 ± 0.07	1.68 ± 0.06	RS4-C	1.51 ± 0.07[a]	1.92 ± 0.06[b]	T: 0.313 I: <0.0001 P: 0.886	PxI: 0.030 IxT: 0.0004
Abdominal noises[3]	Control 1	1.51 ± 0.07	1.34 ± 0.04	RS4-A	1.28 ± 0.05[a]	1.52 ± 0.05[b]	T: 0.681 I: 0.992 P: 0.307	IxT: 0.0006
	Control 2	1.28 ± 0.05	1.26 ± 0.03	RS4-B	1.10 ± 0.02[a]	1.48 ± 0.05[b]	T: 0.137 I: 0.0002 P: 0.926	IxT: 0.0002 PxIxT: 0.027
	Control 3	1.19 ± 0.04	1.35 ± 0.04	RS4-C	1.20 ± 0.04	1.36 ± 0.04	T: 0.199 I: 0.0004 P: 0.987	
Abdominal cramping[3]	Control 1	1.49 ± 0.08	1.34 ± 0.04	RS4-A	1.36 ± 0.07	1.40 ± 0.04	T: 0.751 I: 0.831 P: 0.022	PxI: 0.0004
	Control 2	1.19 ± 0.04	1.13 ± 0.03	RS4-B	1.08 ± 0.03	1.28 ± 0.04	T: 0.963 I: 0.239 P: 0.114	PxI: 0.023 IxT: 0.004
	Control 3	1.17 ± 0.04	1.25 ± 0.04	RS4-C	1.14 ± 0.03	1.32 ± 0.04	T: 0.213 I: 0.0004 P: 0.987	

[1] Unless stated otherwise, values represent mean ± SEM.
[2] P values for treatment (control or fibre)(T), phase of intervention (baseline or intervention)(I), period (P). Means with similar letters were significantly different at P < 0.05.
[3] The mean daily gastrointestinal symptom score represents the sum of symptom intensities (1 = no symptoms to 7 = very severe symptoms).

et al. (2008), who found an increase in *Ruminococcus bromii* with the provision of about 31 g of type 3 RS, and also with Salonen et al. (2014), who demonstrated increased *Ruminococcaceae* phylotypes with the provision of type 3 RS in obese men. Although the potential effects of increased *Ruminococcus* spp. on health are not known, reductions in this genera have been seen with Crohn's disease (Takahashi et al., 2016) and colorectal cancer (Borges-Canha, Portela-Cidade, Dinis-Ribeiro, Leite-Moreira, & Pimentel-Nunes, 2015). The increase that was observed for two OTUs matching to *Ruminococci* and *Faecalibacteria*, two bacterial taxa for which some evidence of health benefits have previously been derived, indicates that RS4-B can induce changes in gut microbiota that might provide benefits to some individuals. Salonen et al. (2014) reported a significant decrease in overall diversity upon RS supplementation in obese individuals, which was not confirmed in the present study. Differences between the two studies include the nature of the RS used (type 3 RS vs. type 4 RS), obese individuals with metabolic syndrome vs. healthy individuals, and microbiota analysis approach (microarray vs. sequencing). Previous *in vivo* studies in animals (Tachon, Zhou, Keenan, Martin, & Marco, 2013) also cannot be expected to mimic effects in humans more than do *in vitro* simulations in anaerobic fermenters (Chung et al., 2016). However, the findings of the present study are supported by *in vitro* work based on batch culture incubations using human faecal inocula, where *Ruminococcus bromii* has been shown to be a key organism for the fermentation of type 3 RS (Ze, Duncan, Louis, & Flint, 2012). The effects of RS supplementation might be affected by long-term dietary habits and underlying microbiota composition. Due to the large inter-individual variation in microbiota composition between individuals, it can be expected that small feeding studies yield different outcomes.

Of significant interest is the finding that RS4-B exhibited a phylum level change in the microbiota of the healthy young adults participating in this study. RS4-B decreased the ratio of

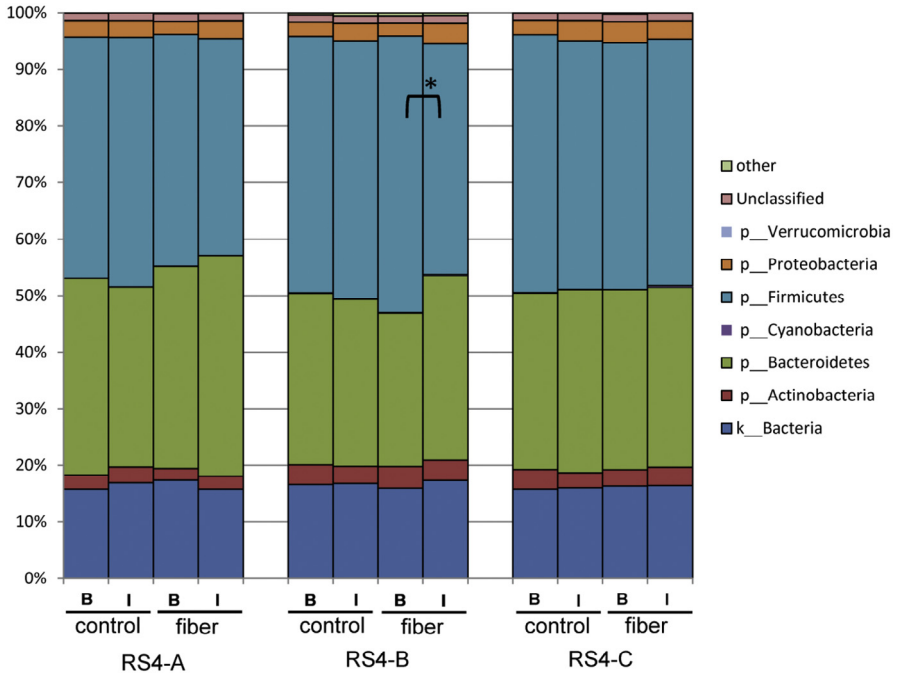

Fig. 2 – Mean proportions of major phyla present in each intervention arm (B – baseline, I – intervention). *Denotes significant change in Firmicutes during RS4-B intervention (P = 0.02).

Firmicutes to Bacteroidetes, suggesting that RS4-B supplementation may have favourable effects on health as an increased ratio has been associated with obesity (Ley, Turnbaugh, Klein, & Gordon, 2006), hypertension (Yang et al., 2015), type 2 diabetes (Remely et al., 2014), and *Clostridium difficile* diarrhoea (Bishara et al., 2013). In the present study, the proportion of Firmicutes decreased by 8%, with a trend for an increase in Bacteroidetes of about 6%. These findings are in agreement with Martinez et al. (2010), who also showed a decrease in the ratio of Firmicutes to Bacteroidetes with type 4 RS as well as a 10% decrease in Firmicutes. Provision of type 2 RS to children also demonstrated a reduction in Firmicutes (Ordiz et al., 2015). Similarly, Holscher et al. (2015) provided 21 g/d of polydextrose and soluble corn fibre, decreasing the ratio of Firmicutes to Bacteroidetes, increasing Bacteroidetesand decreasing Firmicutes by 12 and 13% for the polydextrose and soluble corn fibre, respectively.

Limitations of this study need to be considered. Although more precise measures of transit time, such as radio-opaque markers and wireless motility capsules (WMC), are available, these tools are intended for assessment of gastrointestinal disorders such as gastroparesis and colonic motility disorders (Rao et al., 2011). BSFS is correlated with WMC, whereas stool frequency is not, and is a more practical tool for the evaluation of transit changes in healthy adults (Saad et al., 2010). There is recent evidence to suggest that type 4 RS may impact bacterial levels for a month or more suggesting that the washout may be inadequate for comparisons of bacterial groups (Le Leu et al., 2015). In the present study, there were no significant differences in the OTUs that were found to be affected by fibre supplementation between baseline and washout periods, providing evidence that the washout period was sufficient to achieve a true baseline. The heatmaps (Fig. 3) indicate that any OTUs that changed in prevalence from baseline to end of intervention during the fibre supplementation period did not change to a significant degree during the placebo period, suggesting that these observations represent an intervention effect and not a time effect. Our targeted qPCR analysis with primers for *Bifidobacteria* spp. and LAB did not suggest that fibre supplementation affected their levels. Another significant limitation to the study is that faecal starch analysis was not carried out. It was surprising that RS4-A and RS4-C had no discernible effects on microbiota, suggesting that these products were either digestible or completely resistant to fermentation, and thus determination of faecal starch would provide evidence to answer this question. However, previous research has demonstrated that the consumption of RS4-C resulted in no significant glycaemic response (Haub, Louk, & Lopez, 2012), supporting the premise that this type 4 RS may be resistant to fermentation.

5. Conclusions

Potato starch, chemically modified to resist digestion, varies in its effect on stool form and frequency in healthy individuals. Targeting the traditionally considered "beneficial" genus are major limitations of many published studies, as phylum level changes may have a greater impact on health and disease risk. In this study, RS4-B shifted the ratio of Firmicutes to Bacteroidetes. Further research is needed to determine if RS4-B supplementation impacts phylum level changes and metabolic dysfunction in obese individuals and those with various chronic diseases.

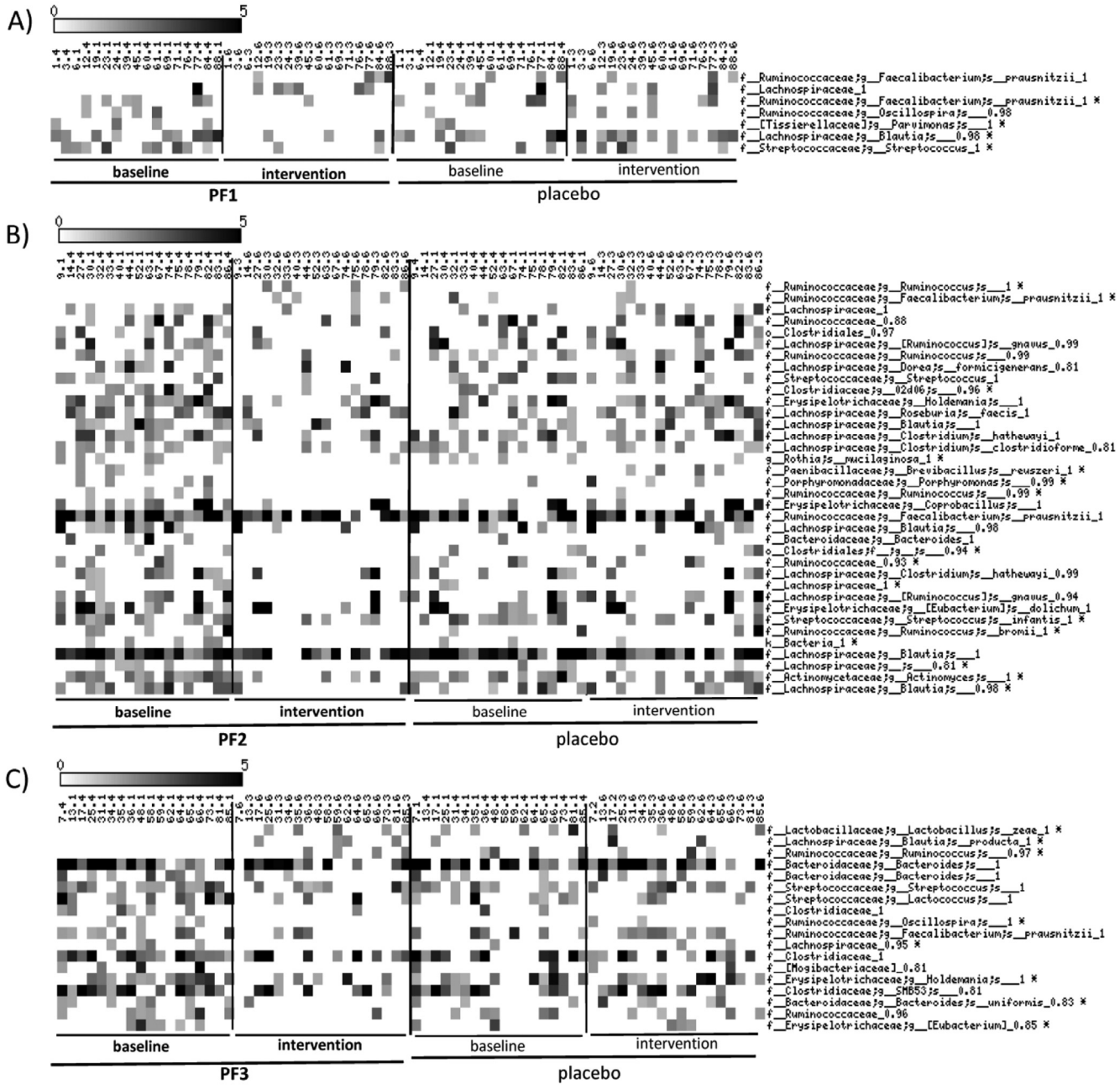

Fig. 3 – Heatmap of Operational Taxonomic Units (OUTs) at 98% level that change during fibre intervention: (A) RS4-A, (B) RS4-B and (C) RS4-C. All OTUs that were significantly different at the 98% similarity level during fibre intervention by Z-test. *After the OTU designation indicates significance ($p < 0.05$) also for TTEST.

Conflict of interest

None.

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Authorship

W.J.D. was responsible for the study design. A.L.F., A.R. and W.J.D. carried out the study. M.U. completed the microbiota analyses. M.C.C., M.U. and W.J.D. completed the statistical analysis and S.W. completed the bioinformatics. W.J.D., A.L.F., V.M., M.U. and M.C.C. wrote the manuscript. W.J.D., A.L.F., M.U., A.R., M.C.C., S.W. and V.M. reviewed and approved the final manuscript.

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