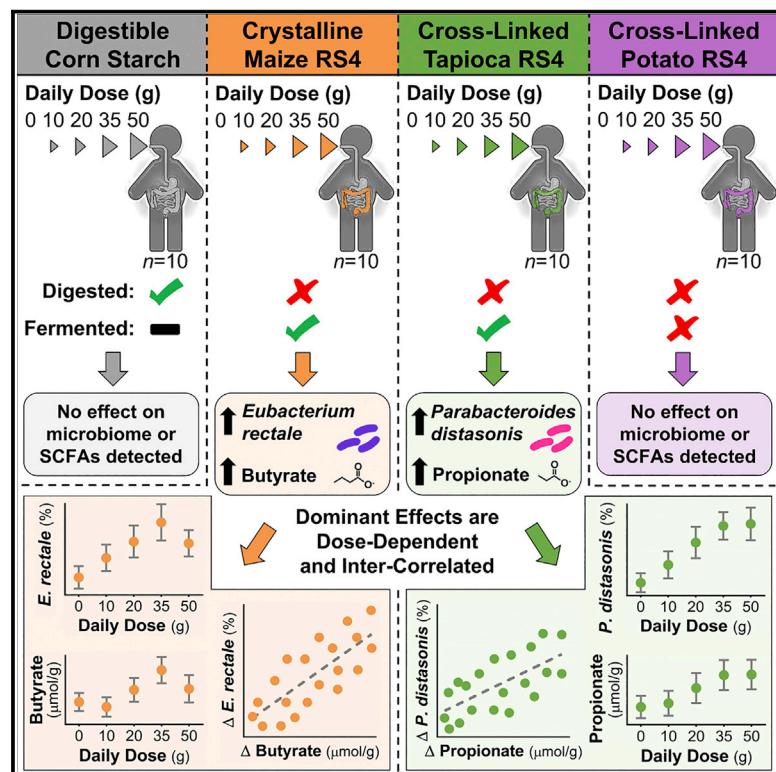


Cell Host & Microbe

Precision Microbiome Modulation with Discrete Dietary Fiber Structures Directs Short-Chain Fatty Acid Production

Graphical Abstract



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In Brief

Deehan et al. show that chemically modified resistant starches with small structural differences induce divergent and highly specific effects on the gut microbiome that direct changes in the output of either propionate or butyrate. Dominant effects were remarkably consistent within treatment groups and dose-dependent with a plateau at 35 g.

Highlights

- Small differences in DF structure distinctly affect the gut microbiome
- Discrete DF structures can direct SCFA output toward either butyrate or propionate
- Dominant effects of DF are dose-dependent and plateau at a daily dose of 35 g/day
- While all responses were individualized, dominant effects were remarkably consistent

Precision Microbiome Modulation with Discrete Dietary Fiber Structures Directs Short-Chain Fatty Acid Production

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SUMMARY

Dietary fibers (DFs) impact the gut microbiome in ways often considered beneficial. However, it is unknown if precise and predictable manipulations of the gut microbiota, and especially its metabolic activity, can be achieved through DFs with discrete chemical structures. Using a dose-response trial with three type-IV resistant starches (RS4s) in healthy humans, we found that crystalline and phosphate cross-linked starch structures induce divergent and highly specific effects on microbiome composition that are linked to directed shifts in the output of either propionate or butyrate. The dominant RS4-induced effects were remarkably consistent within treatment groups, dose-dependent plateauing at 35 g/day, and can be explained by substrate-specific binding and utilization of the RS4s by bacterial taxa with different pathways for starch metabolism. Overall, these findings support the potential of using discrete DF structures to achieve targeted manipulations of the gut microbiome and its metabolic functions relevant to health.

INTRODUCTION

The diverse microbial communities that humans harbor in their gastrointestinal (GI) tract have profound impacts on health. From an evolutionary perspective, the net effect of the gut microbiota is beneficial for the host. However, studies in animal models also suggest a causative role of the microbiome in the development of non-communicable diseases (NCDs) (Valdes et al., 2018). Although the exact factors that drive NCDs are unknown, most NCDs associate with both a Western-type diet and microbiome alterations (dysbioses) characterized by reduced diversity, blooms of opportunistic pathogens, and an imbalanced

ratio of beneficial to detrimental metabolites (Walker and Lawley, 2013). Western-type diets are characterized by high intakes of animal proteins, fats, and refined carbohydrates and low intakes of dietary fibers (DFs) (Makki et al., 2018). Low DF consumption depletes gut microbiome diversity (Sonnenburg et al., 2016) and enhances the production of detrimental metabolites (Russell et al., 2011; Windey et al., 2012); epidemiological and intervention studies identify insufficient DF intake as a factor contributing to NCD development (Reynolds et al., 2019). These observations implicate interactions between DFs and the gut microbiota as a central mechanism in maintaining optimal health.

Plant-based foods deliver a diverse array of DFs to the gut microbiota that favorably shapes its metabolism (Cockburn and Koropatkin, 2016). These include non-starch polysaccharides, oligosaccharides, and resistant starches (RSs), all of which display substantial structural heterogeneity and serve as microbiota-accessible carbohydrates (Deehan et al., 2017). *In vitro* (Reichardt et al., 2018; Yang et al., 2013) and *in vivo* (Baxter et al., 2019; Martínez et al., 2010) studies have shown that structural differences of DFs dictate the microbes involved in their degradation and the effects on the bacterial community, which are often specific yet difficult to predict (Davis et al., 2011; Flint et al., 2015). Fermentation of DF produces short-chain fatty acids (SCFAs; acetate, propionate, and butyrate), which are largely considered beneficial but differ in their physiological effects (Koh et al., 2016). Mechanistic studies in animal models showed beneficial effects of butyrate in maintaining GI barrier integrity, quenching oxygen at the epithelial interface, and exerting immune-modulating effects (Arpaia et al., 2013; Rivera-Chávez et al., 2016), while propionate has been shown to induce satiety through induction of anorectic hormones and intestinal gluconeogenesis (IGN), which also influences glucose metabolism (De Vadder et al., 2014; Psichas et al., 2015). Direct evidence in humans is limited, but butyrate is considered to be anti-carcinogenic and anti-inflammatory (van der Beek et al., 2017), while propionate has been shown to induce satiety (Chambers et al., 2015) and improve glucose metabolism (Chambers et al., 2019; Venter et al., 1990). Therefore, one would predict that a targeted change in the ratio of SCFAs would alter

the physiological, metabolic, and immunological relationship between the gut microbiota and the human host (Tannock and Liu, 2019).

The observations described above point to opportunities by which composition and functions of the gut microbiota could be selectively modulated by DFs. In 2014, Hamaker and Tuncil introduced a conceptual framework that proposes that “discrete structures” within DF molecules (defined as unique chemical structures that align with gene clusters encoded in the genomes of specific microbial species) could be used to obtain predictable changes in microbiota composition to either maintain healthy or correct dysbiotic microbial populations (Hamaker and Tuncil, 2014). Although a promising concept, the authors themselves urged caution that the framework might suffer from an oversimplification of complex ecological interactions within microbiomes (Hamaker and Tuncil, 2014). Species within gut bacterial communities show both functional redundancy, where different species possess the same traits facilitated by horizontal gene transfer (Moya and Ferrer, 2016), and high strain-to-strain variability in important traits (De Filippis et al., 2019). Species also do not function in isolation but form complex networks through mutualistic and competitive interactions (Coyte et al., 2015). In addition, both gut microbiomes and their response to DFs are highly individualized (Martínez et al., 2010; Venkataraman et al., 2016), and they are homeostatic and resilient to change (Coyte et al., 2015). From a more practical perspective, the exact DF dose required for reliable changes, and if such doses are tolerable by modern-day humans, is unknown (Tannock and Liu, 2019). All these factors question whether discrete DF structures could be used to induce targeted and predictable alterations in humans.

In this study, we tested the hypothesis that small discrete differences in the chemical structure of DF can be used to direct changes in fecal microbiota composition and its functions. To achieve this, we performed a randomized controlled trial in humans to compare the effects and dose-response relationships of three type-IV resistant starches (RS4s) on fecal microbiota composition, SCFA profiles, and perceived GI tolerance. RSs were chosen as the DF source because they have well characterized, substrate-specific effects on the human gut microbiome (Baxter et al., 2019; DeMartino and Cockburn, 2020; Martínez et al., 2010), while the use of RS4s with well-characterized chemical modifications allowed the elucidation of specific structure-function relationships between DF and the microbiome. RS further has exciting potential for knowledge translation for the design of food products with high DF doses (Raigond et al., 2015).

RESULTS

Intervention Trial Comparing the Effect of Different RS4s

We performed a randomized double-blinded, placebo-controlled, parallel four-arm dose-response study in 40 healthy individuals ($n = 10$ per arm) to compare the effects of three RS4s (maize, potato, or tapioca derived) and one digestible corn starch (placebo) on the fecal microbiota in humans (Figure 1A; see STAR Methods and Table S1 for supplement specifications). The RS4s differed in chemical structure and granule size (Figure S1). While maize RS4 (VERSAFIBE 2470) was produced through an annealing and acid

treatment of high-amylose maize starch (leading to a restructured starch granule) (Stewart et al., 2018), potato RS4 (VERSAFIBE 1490) and tapioca RS4 (VERSAFIBE 3490) were produced by phosphate cross-linking the native starches (generating inter-starch ester linkages) (Stewart and Zimmer, 2017). Subjects consumed the starches for four weeks to achieve a gradual weekly increase of DF to 10, 20, 35, and 50 g/day, and an equivalent amount of the placebo (Figure 1A).

Protocol adherence rates were high at $98.9\% \pm 2.9\%$ with no differences between groups. Three subjects withdrew from the study because of time constraints (tapioca RS4, $n = 1$) and forgetting the supplement (potato RS4, $n = 2$), thus additional subjects were enrolled and randomly aliquoted to these arms (Figure 1B). Data analyses were limited to the 40 subjects that completed the protocol, which included 20 males and 20 females (5 each per arm) aged 28.4 ± 8.1 years and body mass index of $24.0 \pm 3.2 \text{ kg/m}^2$ (Table S2). Anthropometrics, physical activity, perceived stress, and dietary intake did not change during the intervention, except for additional DF provided as RS4 in the treatment groups on top of the average 18 g/day intake of DF reported by the study cohort (Table S3).

High Doses of RS4 Show Acceptable GI Tolerance

Composite GI tolerability scores (sum of nausea, flatulence, bloating, GI rumbling, abdominal pain, and diarrhea, where higher scores equal poorer tolerance; see STAR Methods) were increased by all treatments, with clear dose responses observed (dose effect $p < 0.0001$, generalized estimating equation [GEE] model; Figure S2A). Maize and tapioca RS4s and the placebo (digestible starch), caused moderate yet significant 1.6- to 2.8-point mean increases in composite tolerability scores at doses ≥ 35 g/day ($p < 0.05$). In contrast, potato RS4 did not affect composite tolerability scores, and no differences were detected between groups (treatment effect $p = 0.19$). Of the 6 GI symptoms assessed, flatulence, bloating, GI rumbling, and abdominal pain were significantly affected by RS4 treatment (dose effect $p < 0.0001$, cumulative link model; Figure S2B).

In a separate survey, the effects of RS4 on bowel habits were assessed (i.e., frequency, consistency, fecal hardness, straining, discomfort, and incomplete evacuation; see STAR Methods). Only potato RS4 induced mild yet significant increases in bowel movement frequency at 50 g/day and decreases in fecal hardness at ≥ 35 g/day, relative to baseline ($p < 0.05$, GEE model; Table S4). Enhanced laxation can be explained by potato RS4 remaining largely unfermented by the gut microbiota (Coulon et al., 2019), because laxation effects are primarily attributable to non-fermentable DFs (McRorie and McKeown, 2017). Overall, these findings, together with findings from other RS interventions trials (Wang et al., 2019b), suggest that modern-day humans without functional GI disorders are able to tolerate high daily doses of RS up to 50 g, as only mild to moderate increases in GI symptoms and minimal changes in bowel habits were detected.

RS4s Differ Markedly in Their Effects on Gut Microbiota Composition

Overall Fecal Microbiota Composition and Diversity

Characterization of fecal bacterial communities was performed by 16S rRNA gene sequencing. Maize and tapioca RS4s increased inter-subject variation (individuality) in microbiome

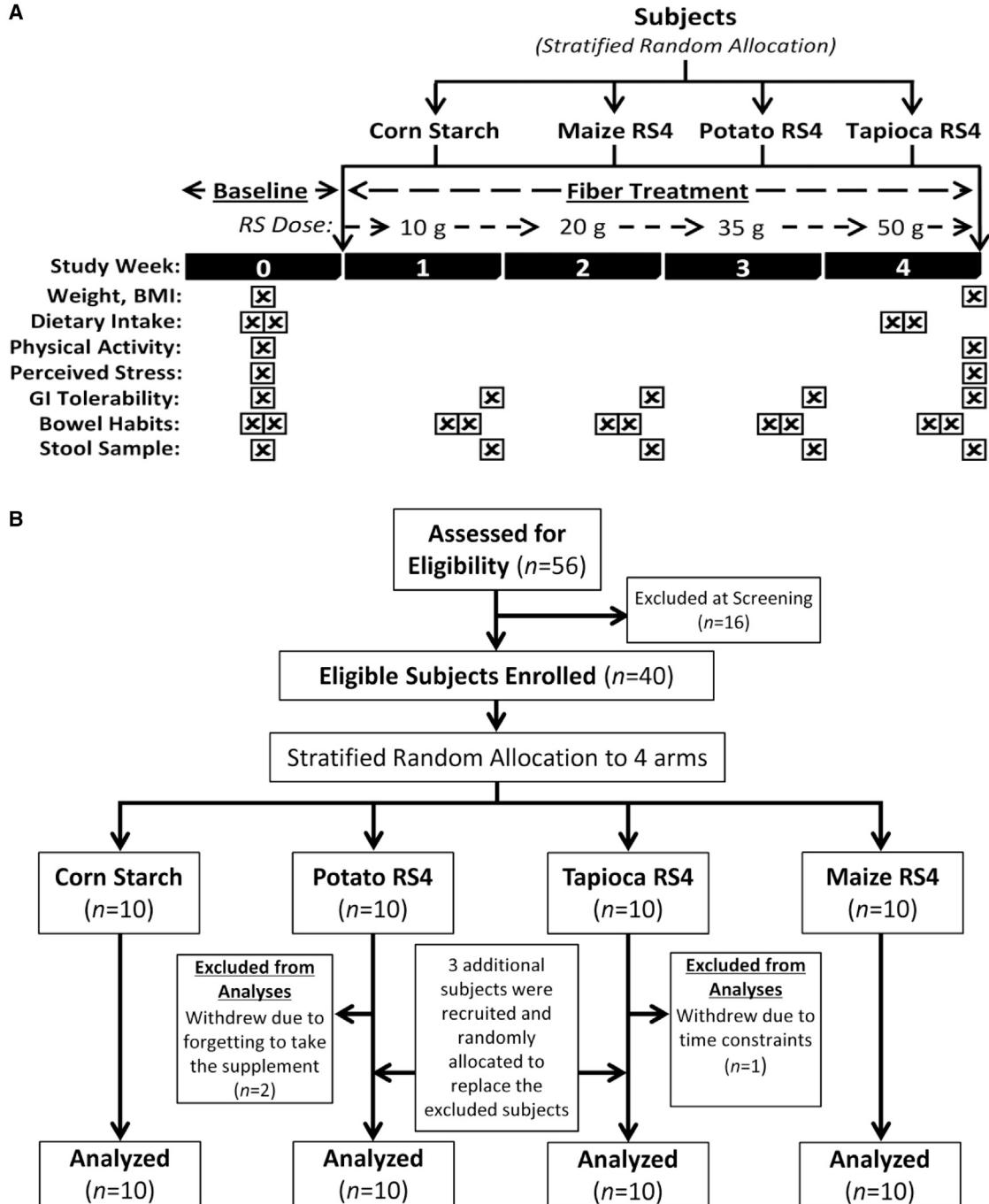


Figure 1. Study Design and Flow Diagram

(A) Study design of the human trial.

(B) Flow diagram of subject recruitment.

See [Tables S2](#) and [S3](#) for subject characteristics.

composition (β -diversity) when compared with baseline and to placebo ($p \leq 0.003$, two-way repeated-measures analysis of variance [rANOVA]; [Figures 2A and S3A](#)). For maize RS4, inter-subject variation increased at doses ≥ 20 g/day. In contrast, for tapioca RS4, inter-subject variation decreased at 10 g/day but then increased at 50 g/day.

We then tested if RS4 consumption induced community-wide effects on microbiota composition. Non-metric multidimensional scaling analysis of Bray-Curtis distances showed differences ($p < 0.05$, PERMANOVA) in the fecal bacterial community of individuals consuming **maize and tapioca RS4s** when compared with baseline and placebo ([Figures S3B and S3C](#)). These

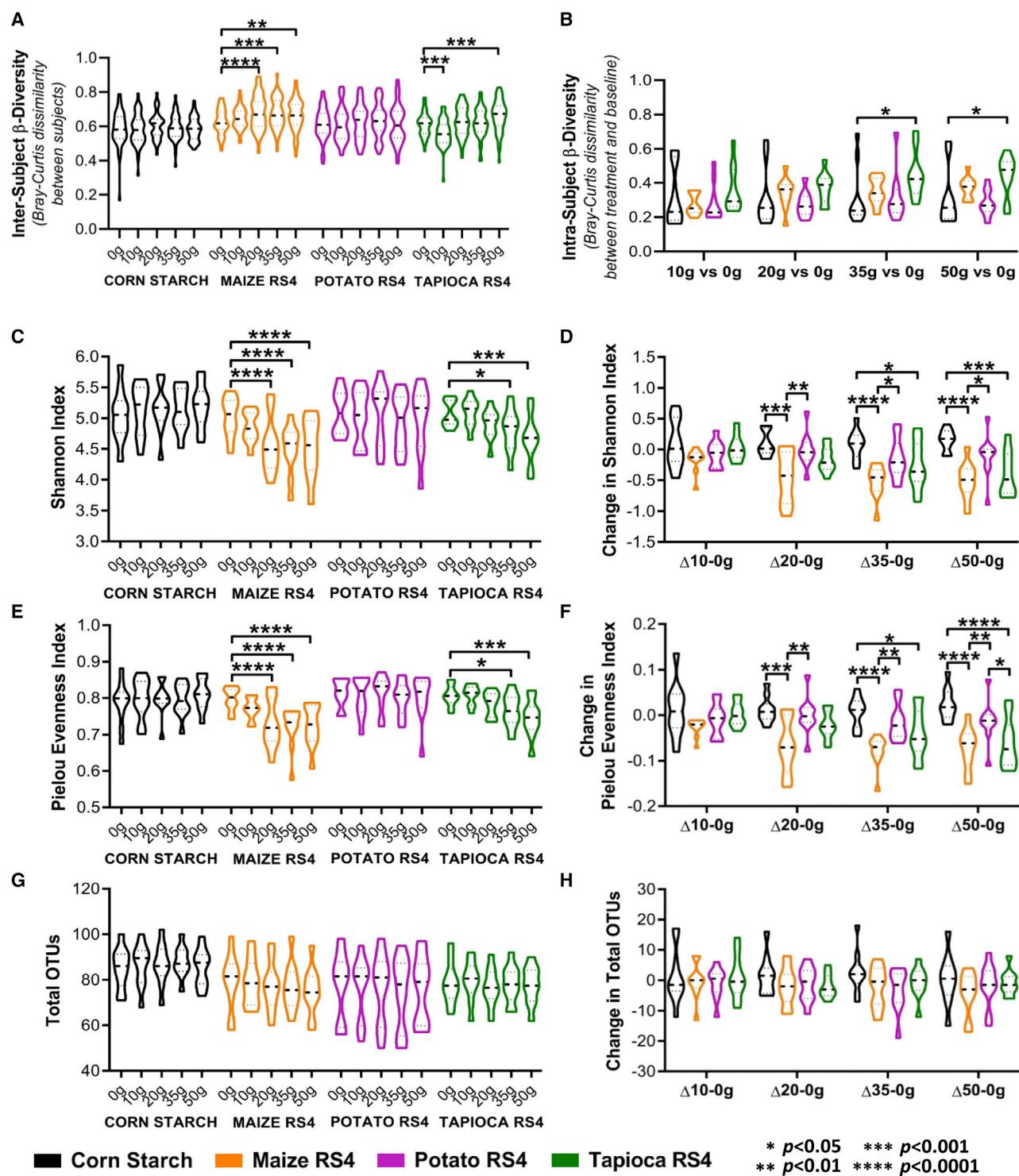


Figure 2. Effects of Different RS4s and Placebo on Fecal Bacterial Diversity

Violin plots of Bray Curtis distances between (A) the fecal microbiomes of subjects at each dose/time point (inter-individual β -diversity), and (B) each subject's fecal microbiome at baseline and during treatment (intra-individual; B was square-root transformed prior to analysis) (see Figure S3 for additional analyses of β -diversity using PERMANOVA). Violin plots showing the α -diversity of the fecal bacterial community at each dose, displayed as (C) Shannon index, (E) Pielou evenness index, and (G) total operational taxonomic units (OTUs). Violin plots showing the shift in diversity at each dose relative to baseline, displayed as (D) Shannon index, (F) Pielou evenness index and (H) total OTUs. Data analyzed using two-way rANOVA (with Holm-Sidak correction).

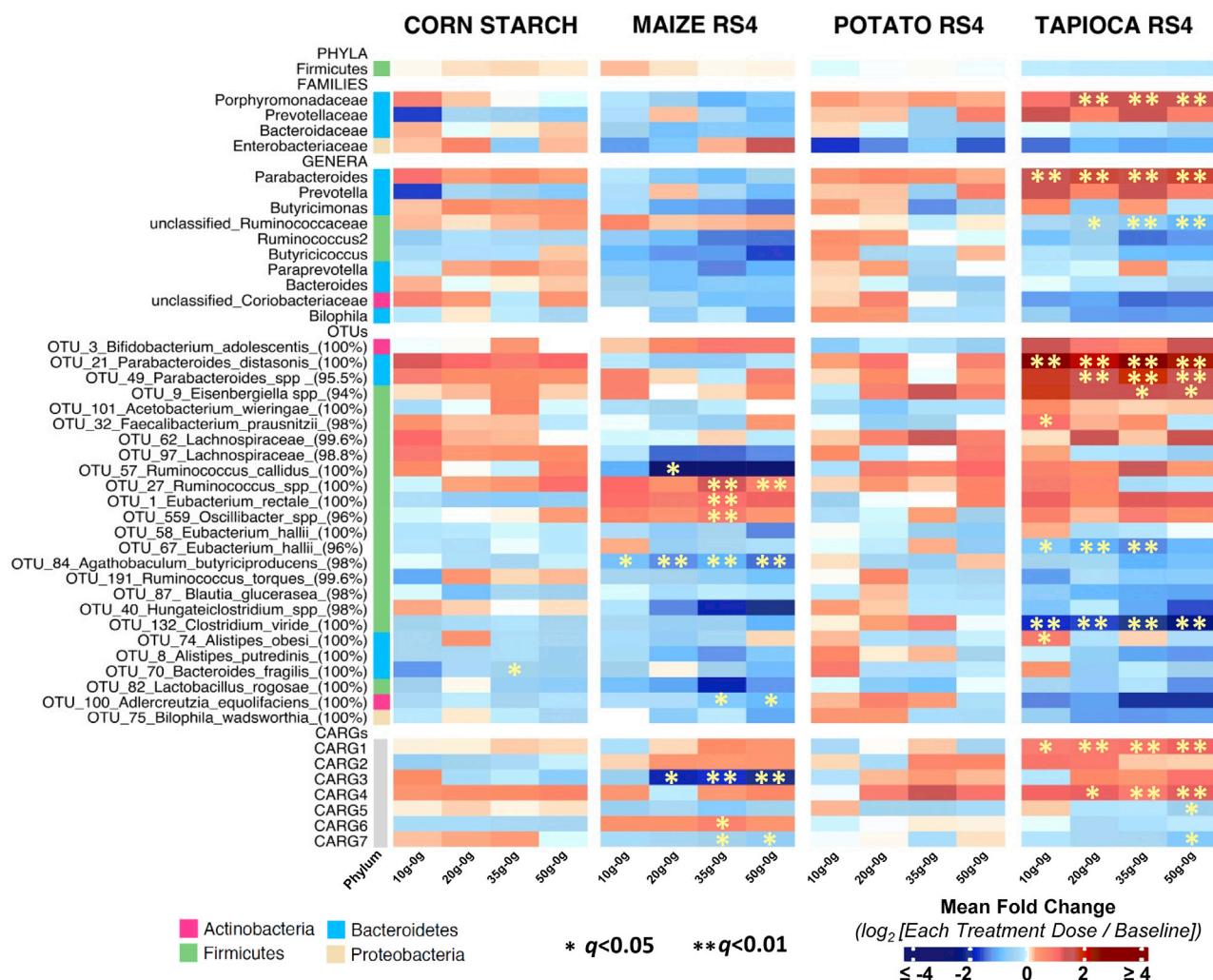


Figure 3. Shifts in the Abundance of Bacterial Taxa and CARGs in Response to RS4s and Placebo

Heatmap of the mean \log_2 -transformed fold change from baseline of phyla, families, genera, and OTUs that showed significant overall dose and/or interaction effects (unadjusted $p < 0.05$; two-way rANOVA) and the identified co-abundance response groups (CARGs; see Table S5 for the same data presented as mean \pm SD, Figure S4 for CARGs identification, and Figure S5 for individual response magnitudes). Statistical significance of changes from baseline at each dose and within each treatment were determined using untransformed data by applying two-way rANOVA (with FDR correction); $q < 0.05$ considered significant.

dissimilarities were likely because of diverging centroids, as no differences were detected in community dispersion ($p > 0.05$, PERMDISP). Tapioca RS4 also induced a significant shift in intra-subject β -diversity at 35 and 50 g/day as compared with placebo (Figure 2B).

Maize and tapioca RS4 reduced the α -diversity (Shannon index) of the bacterial community at ≥ 20 g/day and ≥ 35 g/day, respectively, relative to baseline and placebo ($p < 0.05$, two-way rANOVA; Figures 2C and 2D). For both RS4s, the reduction in α -diversity was because a decrease in community evenness (Pielou evenness index) (Figures 2E and 2F), not a reduction of operational taxonomic units (OTUs; at 98% sequence similarity) within- or between-groups (Figures 2G and 2H).

Overall, the gut microbiome analysis showed that higher doses of maize and tapioca RS4 alter the fecal bacterial community by increasing interpersonal variation, shifting community composition, and reducing community evenness. The placebo

and potato RS4 did not affect β - or α -diversity, supporting the notion that the latter likely remained largely unfermented.

Taxonomic Composition of the Fecal Microbiota

In line with the changes observed in β - and α -diversity, maize and tapioca RS4 changed the relative abundance of bacterial taxa (overall dose/interaction effect $p < 0.05$, Benjamini-Hochberg's false discovery rate [FDR] corrected pairwise comparison $q < 0.05$, two-way rANOVA), while virtually no effects were detected for potato RS4 and placebo (Figure 3; Table S5). The effects were distinct and almost completely substrate-specific. Maize RS4 enriched OTUs related to *Eubacterium rectale* (OTU1), *Oscillibacter* spp. (OTU559), and an OTU within the *Ruminococcaceae* family with 100% similarity to database entries annotated as *Ruminococcus* spp. and *Anaeromassilibacillus* spp. (Guilhot et al., 2017) (OTU27; herein referred to as *Ruminococcus* spp.). In contrast, tapioca RS4 enriched the family *Porphyromonadaceae*, the genus *Parabacteroides*, and OTUs

related to *Parabacteroides distasonis* (OTU21), *Parabacteroides* spp. (OTU49), *Faecalibacterium prausnitzii* (OTU32), and *Eisenbergiella* spp. (OTU9). These enrichments were all substrate specific, although non-significant increases in *E. rectale* and *Oscillibacter* spp. were also observed for tapioca RS4. In addition, *Bifidobacterium adolescentis* (OTU3) showed an enrichment that approached statistical significance ($q < 0.07$) for both maize and tapioca RS4.

Maize and tapioca RS4s also led to reductions in the abundance of taxa. Maize RS4 reduced OTUs related to *Ruminococcus callidus* (OTU57), *Agathobaculum butyriciproducens* (OTU84), and *Adlercreutzia equolifaciens* (OTU100). Tapioca RS4 reduced an unclassified genus of *Ruminococcaceae* and OTUs related to *Eubacterium hallii* (OTU67) and *Clostridium viride* (OTU132) (Figure 3). In contrast to the substrate-specific enrichments by maize and tapioca RS4, many of the reductions induced by maize RS4 were also observed by tapioca RS4, and vice versa, although none reached statistical significance in both groups. Overall, these findings suggest that structural differences between maize and tapioca RS4 selectively increase the fitness of specific OTUs, while reductions in taxa are also detected but appear less specific.

Identification of Co-abundance Response Groups

Bacterial taxa often cooperate during DF degradation, establishing syntrophic interactions through cross-feeding, potentially establishing ecological guilds around primary degraders (Lam et al., 2018). Potential interactions between bacterial taxa in their response to the 50 g/day dose were assessed using co-occurrence network analysis (Tong et al., 2018). The analysis showed that the 55 OTUs most affected by RS4 treatment (dose/interaction effect unadjusted $p < 0.2$, two-way rANOVA) clustered into seven co-abundance response groups (CARGs; Figure S4). As observed with the shifts in OTU abundances, the responses in CARGs were also substrate-specific. Maize RS4 increased the relative abundance of CARG6 ($q \geq 0.011$, two-way rANOVA; Figure 3), which contained *E. rectale* as the only significantly enriched OTU. In contrast, tapioca RS4 increased the abundance of CARG1 ($q \geq 0.001$), which contained *P. distasonis* and *Bifidobacterium* species known to utilize starch (*B. adolescentis* [OTU3] and *B. angulatum* [OTU68]; Duranti et al., 2014), and CARG4 ($q \geq 0.004$), which contained *Eisenbergiella* spp. as the only significantly enriched OTU. Both maize and tapioca RS4 reduced the abundance of CARG7 at ≥ 35 g/day and 50 g/day ($q \geq 0.02$), respectively.

Overall, the CARG analysis supports our conclusion from above that maize- and tapioca-RS4-induced enrichments are highly substrate specific, while the reductions detected are less substrate dependent. Furthermore, CARG1 contained several inter-correlated species of *Parabacteroides* and Actinobacteria (*Bifidobacterium* and *Collinsella*). This suggests that the degradation of tapioca RS4 involves bacterial cross-feeding, which has been described for other RSs (Baxter et al., 2019; Cerqueira et al., 2020; Ze et al., 2012), although the bacterial species involved were different.

RS4 Chemistry Determines Output of Fecal SCFAs

Although RS4 consumption did not alter total SCFA concentrations ($p > 0.1$, two-way rANOVA), the different RS4s varied in their effect on individual SCFAs. Within-group comparisons re-

vealed that maize RS4 selectively increased butyrate concentrations ($p = 0.05$) and relative proportions (percent of total SCFAs) ($p = 0.015$) when compared with baseline, particularly at the 35 g/day dose (Figure 4A). In contrast, tapioca RS4 increased propionate concentrations relative to baseline at 35 g/day ($p = 0.04$). These findings were confirmed by between-group comparisons, which showed that maize RS4 elevated the relative proportion of butyrate ($p = 0.037$, two-way ANOVA treating delta values equally), while reducing the proportion of propionate ($p = 0.001$). Tapioca RS4 increased propionate concentrations when compared with maize RS4 ($p = 0.02$) (Figures 4B and 4C). Neither placebo (digestible starch) nor potato RS4 changed SCFA levels or relative proportions. The latter is in accordance with the absence of *in vivo* fermentation of potato RS4 in rats (Coulon et al., 2019).

Reductions in total and individual branched-SCFAs (BCFAs; isobutyrate and isovalerate) concentrations were detected at doses ≥ 35 g/day when all treatment arms were considered (dose effect $p \leq 0.014$, two-way rANOVA; Figure 4A), but reductions for individual treatments, although detectable, did not reach significance. Significant reductions in the ratio between BCFAs and SCFAs were observed, particularly at the 35 g/day dose, for tapioca RS4 ($p = 0.005$), while reductions approached statistical significance for maize RS4 ($p = 0.07$) relative to baseline (Figure 4D). When comparing between groups, tapioca RS4 reduced the BCFA to SCFA ratio relative to potato RS4 and placebo ($p \leq 0.02$, two-way ANOVA treating delta values equally) (Figure 4E). In summary, it appears that both maize and tapioca RS4s upregulate saccharolytic fermentation with specificity to which SCFA (i.e., butyrate or propionate) was elevated at the expense of BCFAs that are indicative of proteolytic fermentation (Korpela, 2018; Windey et al., 2012).

Effects of RS4s Were Dose-Dependent

To examine RS4 dose-response relationships, we conducted Spearman's correlations between doses (i.e., 0 g/day to 50 g/day) and the abundance of the OTUs that showed the largest increase (>0.75% mean increase in relative abundance), all CARGs, and concentrations of SCFAs (Figure 5A). Consistent with the substrate-specific effects detected above, the dose of maize RS4 positively correlated with *E. rectale* (OTU1; $r_s = 0.34$, $p = 0.015$), CARG6 ($r_s = 0.26$, $p = 0.066$), and butyrate ($r_s = 0.31$, $p = 0.03$), while the dose of tapioca RS4 positively correlated with *P. distasonis* (OTU21; $r_s = 0.39$, $p = 0.005$), CARG1 ($r_s = 0.33$, $p = 0.019$), and propionate ($r_s = 0.28$, $p = 0.049$). Dose-response relationships were not detected for potato RS4 or placebo.

For additional insight into the magnitude of RS4 dose-dependent effects, we plotted the mean abundance or concentration of OTUs (>0.75% increase), CARGs, and SCFAs at all time points, as well as absolute changes of these variables relative to previous time points. As shown in Figures 5B and 5C, all OTUs enriched by maize RS4, as well as the increase of OTU3 (*B. adolescentis*) and CARG6, exhibited a mean that plateaued at 35 g/day. A plateau at 35 g/day was also observed by three OTUs (OTU21, OTU49, and OTU9) enriched by tapioca RS4, as well as OTU3. While the increase of OTU32 (*F. prausnitzii*) with tapioca RS4 peaked at 10 g/day, the increase in CARG1

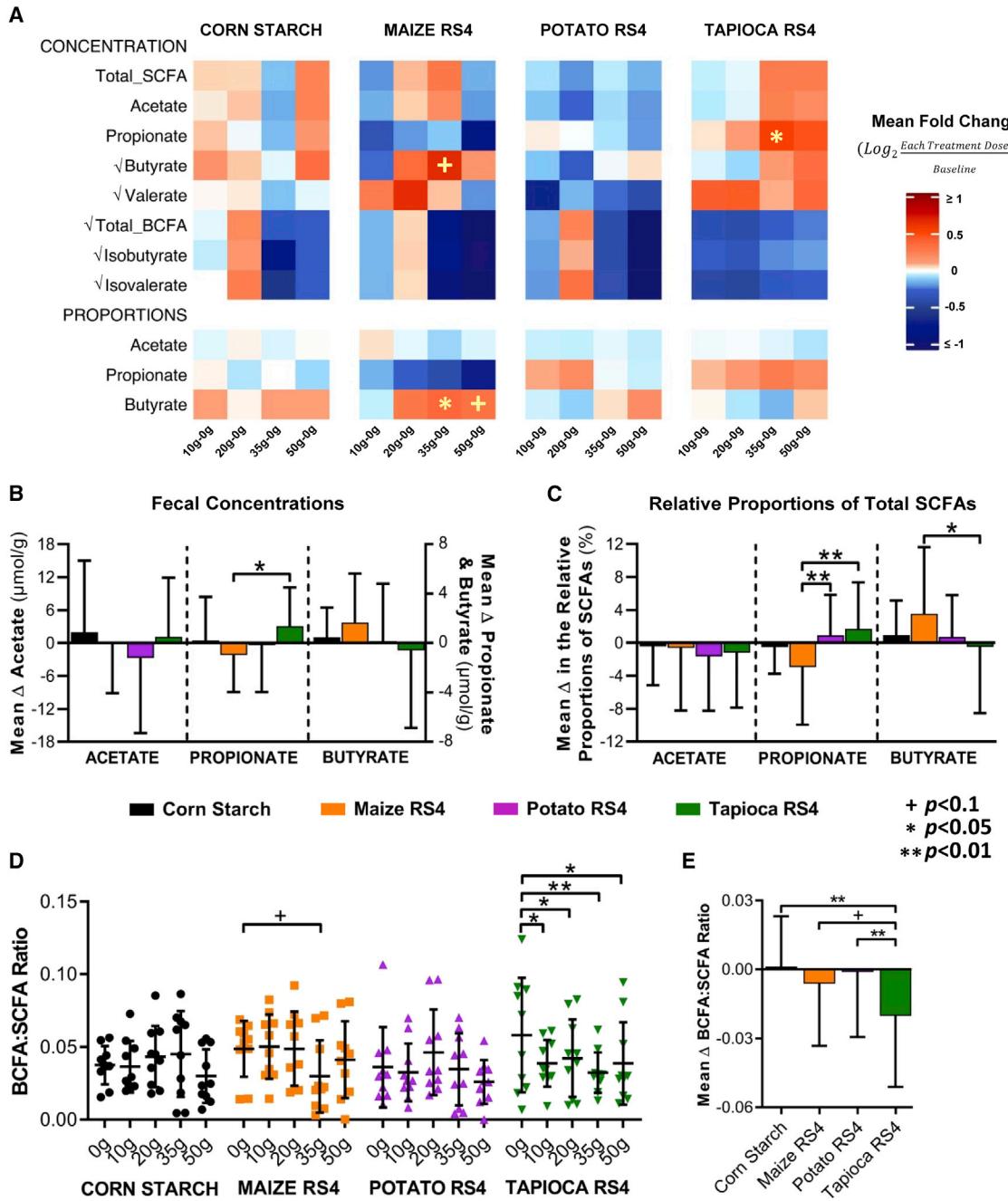


Figure 4. Modulation of Fecal SCFAs through RS4s and Placebo

(A–C) Heatmap of the (A) mean \log_2 -transformed fold change from baseline of SCFA concentrations ($\mu\text{mol/g}$ feces) and the relative proportions of acetate, propionate, and butyrate relative to total SCFAs. Bar plots of the mean (+SD) change from baseline considering all doses for (B) concentrations and (C) relative proportions (%) of SCFAs.

(D and E) Ratio of total branched short-chain to short-chain fatty acids (BCFA:SCFA) at (D) each supplementation dose and the (E) mean (+SD) shift from baseline in BCFA:SCFA considering all doses. Symbols represent individual samples; lines represent mean \pm SD.

Data analyzed for (A and D) using two-way rANOVA (with Holm-Šídák correction) and for (B, C, and E) using ordinary two-way ANOVA (with Holm-Šídák correction) where the four delta values (i.e., $\Delta 10-0 \text{ g}$ to $\Delta 50-0 \text{ g}$) for each subject were treated equally as replicates. $\sqrt{\cdot}$, square root transformed prior to statistical analysis.

did not reach a plateau at any of the doses tested. Interestingly, the increased concentrations of butyrate and propionate induced by maize and tapioca RS4, respectively, also showed means that plateaued at 35 g/day (Figure 5D). Overall, these find-

ings suggest that RS4-induced effects on the gut microbiota are dose dependent, where the average response of most variables detected plateaus at a dose of 35 g/day for maize and tapioca RS4s.

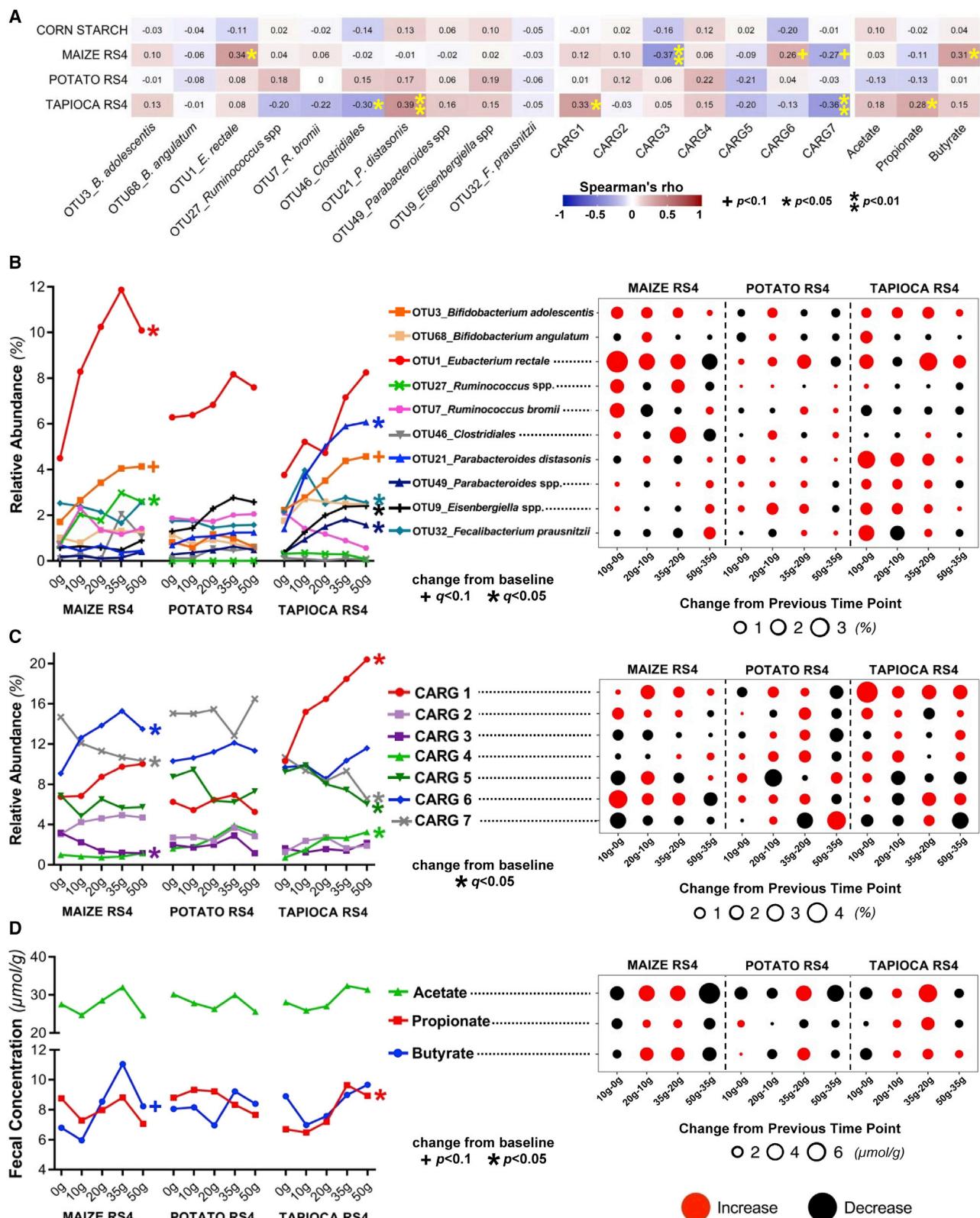


Figure 5. Dose-Dependent Effects of RS4 Treatment on Fecal Bacterial Composition and Function

(A) Dose-response relationships were evaluated using Spearman's correlations between doses (i.e., 0 g/day to 50 g/day) and the abundances of OTUs with mean enrichments >0.75% relative abundance, all CARGs, and concentrations of principal SCFAs ($\mu\text{mol/g}$ feces).

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Individualized Effects of RS4 Consumption

As described in previous RS intervention studies (Martínez et al., 2010; Venkataraman et al., 2016), the effects observed were shown to be individualized, such as increases in *B. adolescentis* (OTU3), *Parabacteroides* spp. (OTU49), and *Eisenbergiella* spp. (OTU9) (Figure S5A). However, some of the detected responses were remarkably consistent. For example, consumption of maize RS4 enriched *E. rectale* (OTU1) in all ten subjects, while CARG6 increased in nine subjects. The main effects of tapioca RS4 were also consistent, leading to an enrichment of *P. distasonis* (OTU21) in all subjects, while CARG1 increased in all but one subject. The magnitudes of these responses were, however, individualized, ranging from an increase of 53% to 535% from baseline for *E. rectale* and an increase of 116% to 21,183% from baseline for *P. distasonis*. Interestingly, we detected a clear co-exclusion pattern between the OTUs classified as *Ruminococcus bromii* (OTU7) and *Ruminococcus* spp. (OTU27), which differed in their response to maize RS4 (Figure S5B), pointing to competitive differences between closely related OTUs as a potential driver for individualized effects.

The increase in butyrate with maize RS4 was also quite consistent (Figure S5C), showing increases in the relative proportion of butyrate in all but two subjects. Tapioca RS4 only increased in the relative proportion of propionate in six subjects. However, one must consider that some of these inconsistencies might arise from fecal SCFA measurements being less sensitive to detect changes in colonic SCFA production because of host absorption (Deehan et al., 2017).

The optimal doses of maize and tapioca RS4 to show maximum effects on OTUs or SCFAs were further individualized. An assessment of individual dose-response curves revealed that although the averages of most effects plateaued at 35 g/day, doses for maximum effect differed among individuals. For instance, the effects of maize RS4 on *E. rectale*, CARG6, and butyrate (Figure S6A) and those of tapioca RS4 on *P. distasonis*, CARG1, and propionate (Figure S6B) continued to be enhanced by the 50 g/day dose for nearly a third of subjects.

Overall, these findings showed that the dominant substrate-specific and dose-dependent effects of the RS4s are remarkably consistent (e.g., CARG1, CARG6, OTU1, and OTU21). However, the findings also emphasize that both the magnitude of compositional and functional responses, as well as the DF dose required to achieve these effects, are individualized.

Selective Effects of RS4s on Microbiota Composition

Explain Responses in SCFAs

To determine whether RS4-induced shifts in the output of SCFAs were linked to the specific effects on bacterial taxa, we conducted Spearman's correlation analyses (Figure 6). Maize-RS4-induced shifts in butyrate proportions were positively correlated with increases in *E. rectale* (OTU1; $r_s = 0.41$, $q = 0.07$), a major butyrate producer (Louis and Flint, 2017), and negatively correlated with increases in *R. bromii* (OTU7; $r_s = -0.49$, $q = 0.02$). In contrast, tapioca-RS4-induced shifts in propionate

proportions were negatively correlated with increases in *Eisenbergiella* spp. (OTU9; $r_s = -0.55$, $q = 0.007$), while being positively correlated with increases in *P. distasonis* (OTU21; $r_s = 0.49$, $q = 0.03$), an important succinate-producing bacterium. Succinate is promptly converted to propionate by other commensal bacteria (Wang et al., 2019), providing an explanation for this association. Several of these correlations within groups were also detectable in the whole dataset; changes in butyrate correlated with *E. rectale* shifts ($r_s = 0.29$, $q = 0.004$) and changes in propionate correlated with *P. distasonis* shifts ($r_s = 0.30$, $q = 0.004$). These findings suggest that shifts in SCFA output are the result of a targeted and structure-dependent effect of the RS4s on microbiota members that possess the pathways to both utilize the RS4 and generate the respective SCFA.

Specific Effects of RS4s Can Be Explained by Selective Bacterial Adherence and Substrate Utilization

To determine the mechanisms that led to the substrate specificity of RS4s, we compared the adherence and utilization ability of 4 strains from amylolytic species that were representative of OTUs enriched by maize and tapioca RS4; *B. adolescentis* IVS-1, *E. rectale* 17629, *R. bromii* L2-63, and *P. distasonis* 8503. The analysis showed that these strains varied in their ability to bind and utilize different RSs (Figure S7). *B. adolescentis* IVS-1, *R. bromii* L2-63, and *P. distasonis* 8503 were all able to adhere to the granules of all RS4s *in vitro*, while *E. rectale* 17629 adhered only to maize RS4 but not potato or tapioca RS4 (Figure 7A). In addition, although all strains were able to utilize maize RS4 for growth, only *B. adolescentis* IVS-1 and *P. distasonis* 8503, but not *E. rectale* 17629 and *R. bromii* L2-63, were able to grow on tapioca RS4 (Figure 7B). In summary, the *in vitro* experiments revealed substrate-specific differences in the adherence and utilization of tapioca RS4 that were in line with our *in vivo* findings, providing a potential mechanism for the specific effects of different RS4s in the human trial.

DISCUSSION

This study revealed that discrete structural differences between DFs can induce substantial yet distinct effects on overall gut microbiota diversity, composition, and functions, leading to selective enrichments of a few bacterial taxa that possess adaptations toward the respective substrates. These compositional responses were linked to directed changes in SCFA output toward either butyrate or propionate. Dominant compositional and functional effects of RS4s were dose dependent and plateaued at a 35 g/day dose in the overall population. Even though all effects showed inter-individual variation, the dominant RS4-induced changes were remarkably consistent. The doses necessary for RS4s to maximize effects on the fecal microbiome were further on average well tolerated.

In ecological terms, DFs constitute resources in the GI tract that support the growth of primary degraders that are able to utilize them directly and microbes that benefit through cross-

(B–D) Line graphs show dose-responses (mean) of the (B) OTUs, (C) CARGs, and (D) SCFAs (see Figure S6 for dose-response plots of each subject treated with maize and tapioca RS4). Bubble plots show changes between doses (e.g., Δ10–0 g and Δ50–35 g), where red and black circles represent positive and negative changes, respectively, and circle size represents the magnitude of change. Statistical significance of changes relative to baseline were determined within each treatment group using two-way rANOVA, where pairwise comparisons were corrected with either FDR (OTU and CARG) or Holm-Sidak (SCFA).

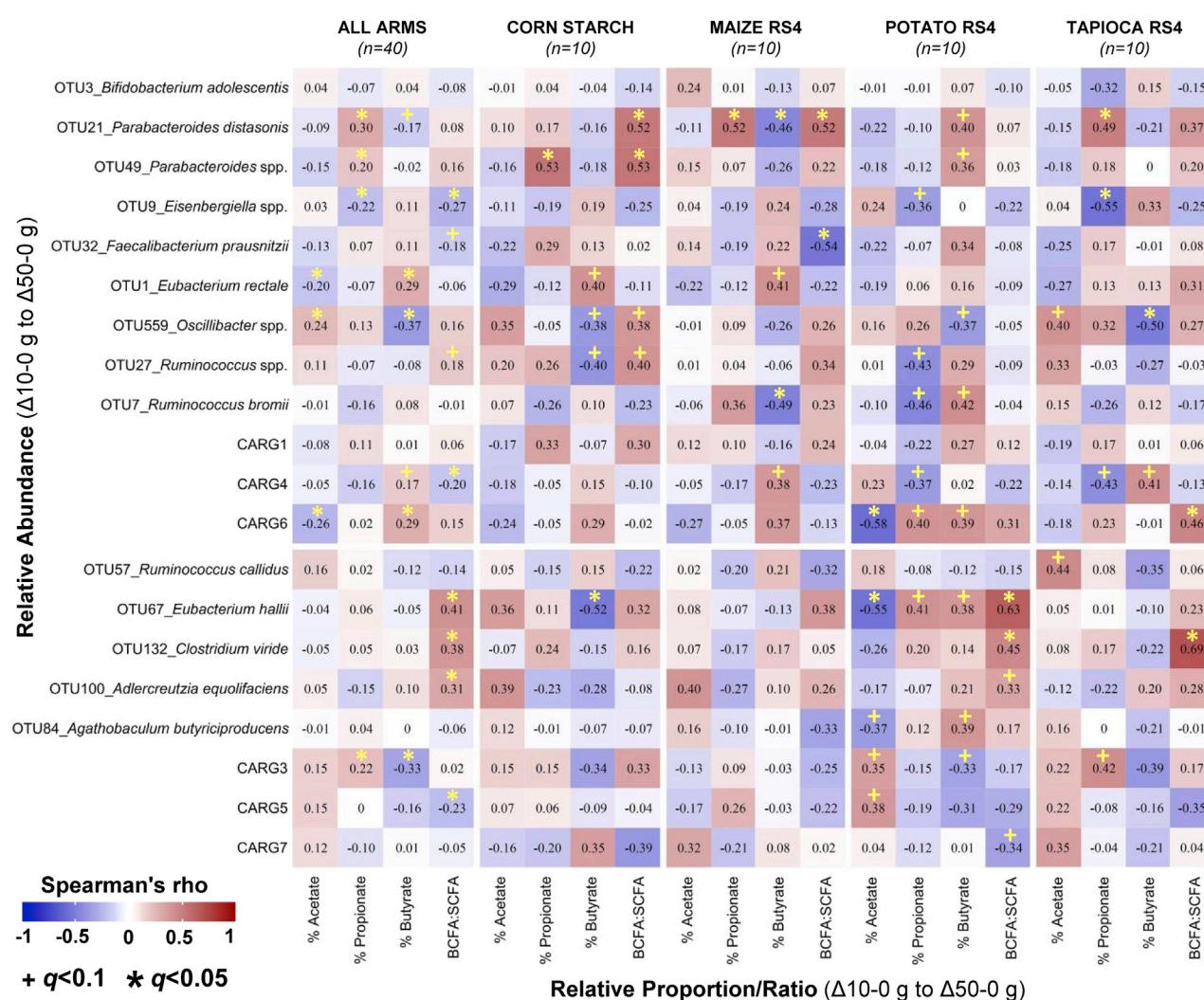


Figure 6. Associations between Shifts of Bacterial Abundances and Changes in the Relative Proportions of SCFAs

Spearman's correlations (with FDR correction) assess associations between shifts in bacterial composition and relative proportions of SCFAs, where all subjects' delta values (i.e., $\Delta 10-0 \text{ g to } \Delta 50-0 \text{ g}$) from the four intervention arms were analyzed together ($n = 40$) and as separate arms ($n = 10$).

feeding of public goods released during degradation. The selective enrichment of only a limited number of bacterial taxa in our study suggests that within the diverse microbiota, only few microbes possess the specialized adaptations needed to competitively access and utilize the molecular structures of maize and tapioca RS4. Accordingly, the microbes enriched are known to efficiently utilize starch (Cockburn et al., 2015; Duranti et al., 2014; Xu et al., 2007; Ze et al., 2012), but they differ in their ability to bind to the RS4s, and also potentially in their ability to access their distinct crystalline and cross-linked structures. The bacteria enriched by maize RS4, such as *B. adolescentis*, *E. rectale*, *Oscillibacter*, and *Ruminococcus* related taxa, were also detected in previous studies that tested granular and retrograded crystalline starches (Abell et al., 2008; Baxter et al., 2019; Martínez et al., 2010; Venkataraman et al., 2016; Vital et al., 2018; Walker et al., 2011). This suggests that re-structuring of the high-amylose maize granule (by annealing and acid treatment) to produce maize RS4 does not alter the microbial affinity to this substrate.

Interestingly, the same bacterial taxa (*B. adolescentis*, *R. bromii*, and *E. rectale*) are also able to selectively colonize RS2 granules, which might constitute an important factor for competitive substrate utilization (Leitch et al., 2007).

In contrast, our findings showed that *E. rectale* was completely unable to bind tapioca RS4 and that *E. rectale* and *R. bromii* were both limited in their growth on the substrate, while *B. adolescentis* and *P. distasonis* both showed good adherence and utilization of tapioca RS4. Phosphate cross-linking acts on the surface of the tapioca granule generating additional inter-starch ester linkages that produce a slightly rough textured surface (Chen et al., 2015). Based on our *in vitro* findings, we speculate that the ester linkages specifically impede surface-attachment by *E. rectale* and resource utilization by *Ruminococcus* species, conferring a competitive advantage to *P. distasonis*. Selective enrichment of *P. distasonis* has also been shown for cross-linked wheat starch (Martínez et al., 2010; Upadhyaya et al., 2016) and butyrate-esterified maize starch (Clarke et al., 2011; Le Lou et al., 2015),

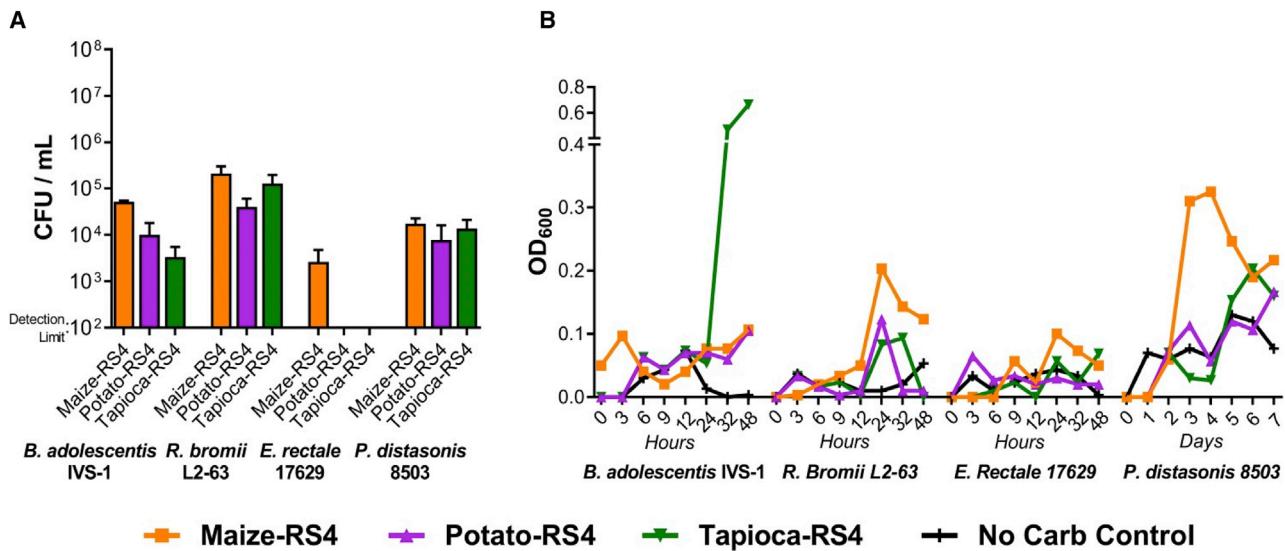


Figure 7. In Vitro Assessment of RS4 Adherence and Utilization by Representative Human-Gut-Derived Amylolytic Bacteria

(A) Total CFUs (CFU/mL) of *Bifidobacterium adolescentis* IVS-1, *Ruminococcus bromii* L2-63, *Eubacterium rectale* 17629, and *Parabacteroides distasonis* 8503 recovered from RS4s after *in vitro* binding assay with the respective RS4 (mean \pm SD).

(B) Growth curves (mean OD₆₀₀) of *B. adolescentis* IVS-1, *R. bromii* L2-63, *E. rectale* 17629, and *P. distasonis* 8503 in YCFA medium containing either 0.2% of the indicated RS4 or no carbohydrate (control).

suggesting that this species possesses specialized traits to access esterified starches. The only species that seemed able to bind and utilize both crystalline and cross-linked starches was *B. adolescentis*, which is in agreement with the consistent enrichment of this species in human intervention trials (Baxter et al., 2019; Martínez et al., 2010; Upadhyaya et al., 2016; Venkataraman et al., 2016). Although differences in chemical modifications of RS4s likely explain the specificity in microbiome response, other factors, such as particle size, might also contribute. This would explain the lack of potato RS4 fermentation in our and previous studies (Coulon et al., 2019; Dahl et al., 2016), as this starch has the largest particle size, which reduces potential attachment sites per particle volume (Tester et al., 2006).

The effects of maize and tapioca RS4 were remarkably specific—most taxa impacted by one RS4 showed no response with the other and several taxa (*P. distasonis*, *F. prausnitzii*, and *Ruminococcus* spp.) even showed opposite responses (Figure 3). This was in contrast to the taxa that decreased in abundance, which were virtually all affected by both RS4s. This finding suggests inhibition as a likely mechanism for these reductions, potentially attributable to colonic environment changes, which are likely less specific than direct competition. A potential mechanism for this inhibition is the increased production of SCFAs, which possess antimicrobial activity themselves (e.g., acetate and propionate; Mani-López et al., 2012) and also acidify the environment inhibiting the growth of pH-sensitive taxa like *Bacteroides fragilis* (Duncan et al., 2009). On a more speculative note, SCFAs upregulate phage production, which might be reducing the abundance of taxa in the community (Oh et al., 2019). It appears that while discrete DF structures enrich specific features of the gut microbiota (including SCFAs); reductions (taxa and BCFAs) are much less specific.

It is of substantial interest that RS4 treatments not only modulated gut microbiota composition but also its metabolism, with strong correlations between compositional shifts and SCFAs that reflect the organisms' metabolic capacities. The RS4-induced taxa that showed strong links to butyrate and propionate shifts, *E. rectale* and *P. distasonis*, respectively, encode the metabolic pathways for butyrate and succinate production, with the latter being readily converted to propionate (Louis and Flint, 2017). Correlations of SCFAs with CARGs (i.e., CARG6 and CARG1) were not significant, which suggests that RS4-induced changes of SCFAs are more dependent on individual taxa and not complex ecological guilds. Although physiological effects of BCFAs have not been elucidated, their reductions do indicate that fermentation of RS4 inhibits colonic protein fermentation, an effect considered beneficial (Korpela, 2018; Windey et al., 2012). In summary, our results suggest that discrete DF structures can be developed to guide the output of specific SCFAs with non-specific reductions of BCFAs.

The dose-response study design allowed us to identify microbiome features that exhibited dose-dependent responses to RS4s and the approximate doses needed to maximize these effects. We can only speculate about the reason for the detected thresholds, but it is possible that the ecosystem is saturated with 35 g/day of RS4, in which taxa reaching maximum growth rates or experiencing limitations in other essential nutrients that limit their expansion. Although our study does not allow direct inferences on health, our findings do provide a basis for tailoring the use of DF to enhance desired effects that are relevant to health. This is pertinent as DF doses used by a vast majority of human trials in the literature are considerably lower than 35 g/day (Armet et al., 2019).

Given the substantial degree of individuality in both gut microbiota composition (Lozupone et al., 2012) and response to diet

(Healey et al., 2017), we were surprised by how consistent and reproducible several of our findings were. This was particularly evident in the enrichments of *E. rectale* and *P. distasonis*, which were observed in every single subject consuming maize and tapioca RS4, respectively. This consistency might have resulted in the directed outputs of butyrate and propionate, which were correlated with these taxa. In addition, comparisons of our findings with those in the literature revealed that the effects of RSs are remarkably reproducible in studies performed in different cohorts, countries, and even continents (Abell et al., 2008; Baxter et al., 2019; Martínez et al., 2010; Venkataraman et al., 2016; Vital et al., 2018; Walker et al., 2011). For example, *B. adolescentis*, *E. rectale*, *R. bromii*, and *Ruminococcus* spp. (OTU27), the latter being 100% identical to seq100 detected by Baxter et al. (2019), have been consistently enriched by crystalline RSs (RS2 and RS3), while phosphate cross-linked RS4s consistently enriched for *P. distasonis* and *B. adolescentis* (Martínez et al., 2010; Upadhyaya et al., 2016). This overlap is remarkable in the light of inter-individual variation, the lack of conserved core-species in human microbiomes (Martínez et al., 2013b), and the low reproducibility in gut microbiome studies because of methodological differences (Costea et al., 2017). It further suggests phylogenetic niche conservatism in bacterial species in relation to the ability to adhere and utilize RS, which implies that within-species strain-level genomic and functional differences and functional redundancies among unrelated species are low with respect to the genes required to utilize RS.

Despite these consistent findings in dominant responses, there was still clear inter-subject variation. Even in taxa consistently enriched, the magnitudes and the doses to achieve maximum changes differed, while several taxa showed an even higher degree of interpersonal variation. The most interesting taxon in this respect was arguably *E. rectale*, which was consistently enhanced with maize RS4 while also showing a very strong response in three individuals with tapioca RS4. In addition, *B. adolescentis* clearly increased in only around a third of individuals, consistent with previous findings (Martínez et al., 2010; Walker et al., 2011). This variation might be because strain-to-strain differences in the ability of *B. adolescentis* to adhere and utilize RS (Crittenden et al., 2001; Duranti et al., 2014). Although overall well tolerated, RS4-induced symptoms were also individualized (Figure S2). Therefore, even though our findings clearly suggest that targeted effects of RS4s can be achieved in a human population, a “one size fits all” approach is unlikely to be universally successful at remodeling dysbiotic patterns, indicating that there is still scope for designing personalized treatments to maximize both health effects and GI tolerance (Kolodziejczyk et al., 2019).

This study revealed important insight for the use of RS4s for microbiota-directed interventions to improve health. In general, maize and tapioca RS4s induce gut microbiota responses that are likely beneficial, enhancing saccharolytic fermentation at the expense of detrimental proteolysis (i.e., SCFA versus BCFA production) without reducing the overall number of taxa. In addition, our findings provide a basis for a more “intelligent” use of maize and tapioca RS4 to achieve targeted manipulation of dysbiotic gut communities to yield specific health endpoints. This strategy could be further extended to fecal microbiota transplants with an aim to provide substrates for selective niche op-

portunities of specific bacterial species predicted to provide benefits. For instance, maize RS4 might be applicable for correcting the dysbiosis seen in type 2 diabetes, where *E. rectale* has been shown to be low in abundance (Qin et al., 2012), a species positively associated with improved glycemic control (Martínez et al., 2013a; Zeevi et al., 2015). Furthermore, the immunoregulatory properties of butyrate (Koh et al., 2016) makes maize RS4 a candidate for the treatment and/or prevention of colorectal cancer, inflammatory bowel disease, and obesity-associated inflammation. Tapioca RS4, on the other hand, could be used to correct dysbiotic communities where *P. distasonis* abundance is reduced, such as obesity and non-alcoholic fatty liver disease (Del Chierico et al., 2017; Verdam et al., 2013). To this end, treatment with live *P. distasonis* has recently been shown in mice to decrease high-fat-diet-induced weight gain, hyperglycemia, and hepatic steatosis (Wang et al., 2019). Propionate could be further targeted by tapioca RS4 for treatment of insulin resistance and obesity, owing to its upregulation of IGN (De Vadder et al., 2014) and anorectic hormones (Psichas et al., 2015). Mixtures of maize and tapioca RS4 could be used to increase both butyrate and propionate production simultaneously, which would be particularly relevant for the treatment of obesity and its dysregulated immunometabolism. Given the technological attributes of RSs in the production of flour-based foods (Raigond et al., 2015), they could be readily incorporated into medical foods for specific patient populations and the general food supply.

Overall, this study provides critical evidence in support of a mechanistic framework for intelligent manipulation of the colonic microbiota with discrete DF structures (Hamaker and Tuncil, 2014). Our correlation analyses indicate that key aspects of the framework apply to the competitive constraints of a human microbiome because DF structures do in fact align with phenotypes of specific microbes that differ in their metabolic pathways, directing the output of physiologically relevant metabolites. The ability to employ small differences in DF chemical structure to achieve substantial, highly selective, and tractable effects on the gut microbiome paves the way for the development of precision approaches that could involve designer carbohydrates that target functional outputs relevant to health. The notable dose dependency of DF-induced effects established in this study permits a more systematic and precise modulation of the microbiome, information that is for most part lacking in the field. Although approaches could be optimized through personalization, the consistency of our main findings and their high reproducibility among other published studies suggests that microbiome-modulating strategies based on discrete DF structures could be successfully applied on a population-wide basis.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.chom.2020.01.006>.

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AUTHOR CONTRIBUTIONS

Conceptualization and Funding Acquisition, E.C.D. and J.W.; Methodology, E.C.D., C.C.C., and J.W.; Investigation, E.C.D., C.Y., C.C.C., and L.T.; Formal Analysis, E.C.D., M.E.P.-M., N.K.N., Z.Z., J.A.B., and J.W.; Project Administration, E.C.D.; Supervision, J.W.; Writing – Original Draft, E.C.D., M.E.P.-M., and J.W.; Writing – Review & Editing, all authors.

DECLARATION OF INTERESTS

J.W. has received research funding and consulting fees from industry sources involved in the manufacture and marketing of DFs, including Ingredion, which supported this study and commercializes all starches used. J.W. is further a co-owner of Synbiotic Health, a developer of symbiotic products. These interests did not influence his judgement or presentation of study findings. All other authors declare no conflict of interest.

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WEB RESOURCES

ClinicalTrials.gov, www.clinicaltrials.gov/ct2/show/NCT03255603

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
<i>Bifidobacterium adolescentis</i> IVS-1	Krumbeck et al. 2018	PMID: 29954454
<i>Eubacterium rectale</i> DSM 17629	Barcenilla et al. 2000	PMID: 10742256
<i>Ruminococcus bromii</i> L2-63	Ze et al. 2012	PMID: 22343308
<i>Parabacteroides distasonis</i> ATCC 8503	Sakamoto and Benno, 2006	PMID: 16825636
Biological Samples		
Healthy, human fecal samples	This paper	Walter Lab
Chemicals, Peptides, and Recombinant Proteins		
Agar	Fisher	Cat# BP1423
K ₂ HPO ₄	Fisher	Cat# P288
KH ₂ PO ₄	Fisher	Cat# BP362
NaCl	Fisher	Cat# BP38-212
MgSO ₄	Sigma	Cat# M7506
CaCl ₂	Fisher	Cat# C79
Biotin	TCI America	Cat# B0463100MG
Cobalamin	TCI America	Cat# C0449100MG
p-Amino benzoic acid	TCI America	Cat# A026925G
Folic acid	MP Biomedicals	Cat# ICN10172505
Pyridoxamine	ACROS Organics	Cat# AC436250010
Thiamine HCl	TCI America	Cat# T018125G
Riboflavin	Alfa Aesar	Cat# AAA1176414
Hematin	Carbosynth	Cat# FH44826
L-histidine	ACROS Organics	Cat# AC411730250
Tryptone	VWR	Cat# CA90000-286
Yeast extract	Fisher	Cat# BP1422-500
Na ₂ CO ₃	Fisher	Cat# S233
L-cysteine	VWR	Cat# CAAAAL06328-14
Acetic acid	Fisher	Cat# 351270-212
Propionic acid	Fisher	Cat# A258-500
Isobutyric acid	Alfa Aesar	Cat# AAL04038AE
Isovaleric acid	Alfa Aesar	Cat# AAA18642AK
Valeric acid	TCI America	Cat# V000325ML
Resazurin	TCI America	Cat# R02031G
NaOH	Fisher	Cat# SS256
D-(+)-Glucose	Fisher	Cat# BP350
D-(+)-Maltose	VWR	Cat# 97062-608
D-(+)-Galactose	Sigma	Cat# G0750
D-(+)-Fructose	Fisher	Cat# L95
Critical Commercial Assays		
QIAamp DNA Stool Mini Kit	QIAGEN, 40724 Hilden, Germany	www.qiagen.com
Deposited Data		
Raw sequence data (16S rRNA)	This paper	NCBI BioProject: PRJNA560950 (Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
16S rRNA-Forward Primer 784F: 5'-RGGATTAGATACCC-3'	Krumbeck et al. 2018	PMID: 29954454; genomics.umn.edu
16S rRNA-Reverse Primer 1064R: 5'-CGACRRCCATGCANCACCT-3'	Krumbeck et al. 2018	PMID: 29954454; genomics.umn.edu
Software and Algorithms		
Canadian Automated Self-Administered 24-Hour Dietary Assessment Tool (Canada ASA24-2016)	National Cancer Institute and Health Canada	asa24.ca/index.html
PRISM v. 8.3	GraphPad	www.graphpad.com
R Stats Software v. 3.5.1	R Core Team	www.r-project.org
QIIME v. 1.9.1	Caporaso et al. 2010	qiime.org
QIIME2 v. 2018.6	Hall and Beiko, 2018	qiime2.org
Vegan v. 2.4-4	Oksanen et al., 2017	cran.r-project.org/web/packages/vegan/ vegan.pdf
FastX-toolkit	Hannon Lab	hannolab.cshl.edu/fastx_toolkit/
Illumina utils	Merem Lab	merenlab.org/software/
Usearch v. 10	Edgar, RC	PMID: 20709691
Ribosomal Database Project	Michigan State University	PMID: 17586664
EzBioCloud	Chun Lab	PMID: 28005526
NCBI blastn	US National Library of Medicine	blast.ncbi.nlm.nih.gov/Blast.cgi
Other		
AMIOCA™ Powder TF (Placebo)	Ingredion Inc, Bridgewater, NJ 08807, USA	www.ingredion.com
VERSAFIBE™ 2470 (Maize RS4)	Ingredion Inc, Bridgewater, NJ 08807, USA	www.ingredion.com
VERSAFIBE™ 1490 (Potato RS4)	Ingredion Inc, Bridgewater, NJ 08807, USA	www.ingredion.com
VERSAFIBE™ 3490 (Tapioca RS4)	Ingredion Inc, Bridgewater, NJ 08807, USA	www.ingredion.com
HI-MAIZE® 260 (Maize RS2)	Ingredion Inc, Bridgewater, NJ 08807, USA	www.ingredion.com
Unmodified Potato Starch (Potato RS2)	Bob's Red Mill, Milwaukie, OR 97222, USA	www.bobsredmill.com

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jens Walter (jenswalter@ucc.ie). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Subjects

This study was prospectively registered with [ClinicalTrials.gov](https://clinicaltrials.gov) (identifier: NCT03255603) and was conducted at the University of Alberta Human Nutrition Research Unit in Edmonton, Canada between September 2017 and February 2018 in accordance with the principles of the Declaration of Helsinki. All procedures involving human subjects were approved by the Health Research Ethics Board of the University of Alberta (Approval Number: Pro00069884).

Written informed consent was obtained from all study subjects prior to enrollment into the study. Study subjects included healthy males and pre-menopausal, non-pregnant or lactating females aged 18 to 50 years that were recruited using campus-wide flyers, mailings to specific Listservs, local events, and word of mouth. Exclusion criteria included: (1) patient history of GI diseases or surgeries; (2) use of antibiotics 3-months prior to the start of the study; (3) chronic use of anti-hypertensive, lipid-lowering, anti-diabetic, analgesic, or laxative medications; (4) use of probiotic or prebiotic supplements; (5) intolerance to corn, potato, or tapioca; (6) vegetarian; (7) smoking; (8) alcohol intake ≥ 8 drinks/week; (9) more than 5 h of moderate-vigorous exercise per week. After exclusions and replacements of subjects that withdrew because of time constraints (Tapioca RS4, n = 1) and forgetting to take the supplement (Potato RS4, n = 2), a total of 40 adult subjects (n = 10 per arm), including 20 male and 20 female with a mean age of 28.4 ± 8.1 years, completed the dietary intervention and were included in final data analyses (Table S2).

METHOD DETAILS

Experimental Design and Randomization

The trial used a randomized, double-blinded, placebo-controlled, parallel 4-arm, 4-week dose escalation design (Figure 1A). Random allocation was done using stratified random assignment based on sex, with 5 males and 5 females being assigned to each of the three treatment arms (3 structurally distinct RS4s) or the placebo arm (digestible corn starch). Two separate random allocation sequences (male and female sequence) were generated (by a study investigator not involved in subject recruitment and allocation) using the website Randomization.com with four randomly permuted blocks (www.randomization.com), and then concealed using two lists of randomly generated codes. Sample size ($n = 10$ per arm) was determined by referencing previous studies that successfully assessed the effect of DF on GI microbiome composition and GI symptoms (Calame et al., 2008; Cherbut et al., 2003; Martínez et al., 2010; Mego et al., 2017; So et al., 2018; van den Heuvel et al., 2004).

Five weekly clinic visits were held for each subject (Figure 1A). Potential subjects completed an initial telephone pre-screening followed by a baseline visit (week 0) to confirm eligibility. Upon enrollment, subjects were assigned to the next available randomization code by study investigators blinded to the predetermined allocation sequences, and then instructed to consume the corresponding RS4 or placebo supplement daily for four weeks, with the DF dose provided strictly as RS being raised weekly (Week 1: 10 g/d, Week 2: 20 g/d, Week 3: 35 g/d, Week 4: 50 g/d). The starches were administered as a supplement, divided into 2 to 3 servings, and then incorporated into water or other preferred drinks and foods without cooking.

Dietary Supplementation

The three RS4s and placebo (digestible starch) were all manufactured and provided by Ingredion Incorporated (Bridgewater, NJ, USA) as single batches. Supplement specifications, including their chemical structure, are provided in Table S1 and Figure S1. Maize RS4 (VERSAFIBE™ 2470) is a high-amylose maize starch subjected to acid hydrolysis to remove nonenzymatic-resistant material, and then to annealing treatments in order to reorganize and increase the stability of the native granule structure (Brumovsky and Thompson, 2001). Potato RS4 (VERSAFIBE™ 1490) and Tapioca RS4 (VERSAFIBE™ 3490) are native potato and tapioca starches subjected to a phosphorus oxychloride treatment that reduces digestibility by cross-linking starch molecules at the surface of the starch granule, creating a slightly rough surface in comparison with the native starch (Chen et al., 2015). The native corn starch used as the placebo is AMIOCAT™ powder starch, a high amylopectin starch that should be rapidly digested and absorbed proximally in the small intestine, which prevents its availability for microbial fermentation in the colon, making it an ideal placebo when characterizing the microbial response to RS.

The supplements were identical in appearance (white powders), and weekly doses were provided in sealed opaque bags that contained individually packaged, ready-to-use daily sachets that provided the desired doses of DF (i.e., 10 g/d to 50 g/d). The absolute amount was dependent on each supplement's DF content based on measurements with AOAC 2009.01 and adjusted for moisture content. The amount of placebo (digestible corn starch) packaged was equal to the mean amount of the RS4s used in the three treatment arms. Packaging, coding (i.e., 'Starch 1' to 'Starch 4'), and the unblinding upon completion of data collection were carried out by an individual not involved in the study. Subjects were instructed to return all provided sachets at their weekly visits, where the remaining portion of unconsumed supplement was weighed to assess treatment protocol adherence.

Lifestyle and Anthropometric Assessments

Subjects were asked to maintain their habitual diet and physical activity level during the study, and instructed to avoid foods known to cause GI symptoms, such as cabbage, artichokes, onions, beans, lentils, wheat bran, prunes, and plum juice (Cherbut et al., 2003). To assess dietary intake maintenance, subjects completed two 24 h recalls, both at baseline and during week 4, using the Canadian version of the Automated Self-Administered 24 h Dietary Assessment Tool (ASA24-Canada-2016), a method perceived to be less burdensome than other 24 h recall methods (Csizmadi et al., 2007; Thompson et al., 2015). Anthropometrics (height, weight, and body mass index [BMI]), physical activity (7-day total metabolic equivalent of task score), and perceived stress (1-month total perceived stress score) were assessed at baseline and during week 4 using the validated International Physical Activity Questionnaire and Perceived Stress Scale, respectively (Cohen and Williamson, 1988; Mäder et al., 2006).

GI Tolerability and Bowel Habit Assessments

GI tolerability was assessed during all five clinical visits using a questionnaire to rate the severity of specific GI symptoms six days prior to the visit: nausea, GI rumblings, abdominal pain, bloating, flatulence, and diarrhea. The severity of each symptom was reported on a 3-points scale, with '0' denoting 'no symptoms/no more than usual', '1' denoting 'somewhat more than usual', and '2' denoting 'much more than usual'. A composite GI tolerability score was then calculated as the sum of each individual symptom score, with a range from 0 to 12 (representing complete tolerance and poor tolerance, respectively) (Maki et al., 2013; Stewart et al., 2018). Subjects also completed a bowel movement habit diary at baseline and over the two days preceding each clinic visit, recording bowel movement frequency, fecal consistency using the Bristol Stool Scale (scale of 1 [hard] to 7 [liquid]), and subjectively rating perceived fecal hardness (scale of 1 [soft] to 4 [very hard]), straining during bowel movement, discomfort during bowel movement, sensation of incomplete evacuation (all with a scale of 1 [none] to 4 [severe]) (Maki et al., 2013). A mean daily score was calculated for each bowel movement habit prior to statistical analyses.

Fecal Microbiome Sequencing

Subjects collected fecal samples at baseline and the end of each week using a stool specimen container (Fisher, Canada), and delivered them to the investigators within 4 h of defecation for immediate processing. Aliquots of fecal material, 1:10 fecal homogenates in phosphate-buffered saline (for DNA extraction), and 1:5 fecal homogenates in 5% phosphoric acid (for SCFA analysis) were immediately frozen (-80°C) and stored until further processing. Bacterial DNA was extracted from fecal homogenates as previously described (Martinez et al., 2010) with slight modifications: a reduction in the lysis step to 15 min and an elimination of the InhibitEX tablet provided in the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) were performed as recommended by Costea and colleagues (Costea et al., 2017).

Composition of the bacterial community in fecal samples was characterized using 16S rRNA gene amplicon sequencing. PCR targeting the V5-V6 region of the 16S rRNA gene with primers 784F [5'-RGGATTAGATACCC-3'] and 1064R [5'-CGACRRCCATGCAN CACCT-3'], and subsequent amplicon sequencing (Illumina MiSeq platform v3 kit producing 300-bp paired-end sequences) was performed at the University of Minnesota Genomics Center, with DNA from all 200 fecal samples being included in a single sequencing run. Amplicon sequencing produced a total of 14,637,282 raw sequences (average = 73,554; minimum = 34,324 and maximum = 109,526). To make the dataset manageable, R1 and R2 fastq files were randomly subsampled, based on the sample with the lowest amount of reads, to obtain 30,000 matching reads using an in-house python script. Raw reads were trimmed to 210 bases long using FASTX-toolkit (hannonlab.cshl.edu/fastx_toolkit/index.html). R1 and R2 ends were quality filtered and paired using the merge-illumina-pairs application from Illumina utils (Eren et al., 2013). Sequences that didn't meet the quality criteria (p value of 0.03, enforced Q30 check, perfect matching to primers, and no ambiguous nucleotides allowed) were discarded. One sample collected after consuming the 50 g/d dose of Tapioca RS4 did not amplify; therefore data from the subject's 35 g/d dose was carried forward. After trimming and quality filtering, a total of 4,523,790 paired sequences were obtained (average = $22,619 \pm 443$; minimum = 21,103 and maximum = 23,877). Sequences from all samples were compiled and dereplicated using Usearch v.10 (Edgar, 2013). Subsequently, singletons were discarded, chimeras removed, OTUs clustered at 98% identity, representative sequences for OTUs were selected and an OTU table was generated using Usearch v.10 (Edgar, 2013). Non-chimeric sequences were binned by sample/subject and submitted to Ribosomal Database Project Classifier (Wang et al., 2007) for taxonomic assignment. OTUs were assigned taxonomy using Silva database (release 132) (Westram et al., 2011) and sequence identity confirmed using NCBI blastn (Altschul et al., 1990), EzBioCloud (Yoon et al., 2017), and Ribosomal Database Project Seqmatch (Michigan State University, 2016). Counts were transformed to relative abundance. Taxa with a mean relative abundance of $\leq 0.10\%$ were removed from the dataset prior to statistical analyses. Diversity analyses were performed using Qiime (Caporaso et al., 2010) and Qiime2 (Hall and Beiko, 2018).

To determine groups of interacting OTUs in their response to RS4 supplementation, CARGs were determined from the top OTUs impacted by the dietary intervention (dose/interaction effect unadjusted p value less than 0.2; 2-way rANOVA) (Tong et al., 2018). Among the three treatment groups (i.e., not including placebo), Spearman's correlation analyses were performed between the shifts in these OTUs (i.e., 0 g/d to 50 g/d) to construct a correlation matrix. Hierarchical clustering was then performed on the matrix and a tree was built based on this matrix using the Ward algorithm. Differences between distinct branches of the Hierarchical tree, and thus individual CARGs, were determined by PERMANOVA (using a less-stringent cut-off of $p \leq 0.1$) and by visual inspection of the Hierarchical tree in order to separate OTU clusters that displayed clear differences in their response (Tong et al., 2018). Relative abundance of each CARG was calculated as the sum of the OTUs within each CARG prior to statistical analyses.

Fecal SCFA Quantification

SCFAs were analyzed at the Agricultural, Food and Nutritional Science chromatography core facility of the University of Alberta as previously described (Jin et al., 2019), with modifications. Briefly, previously acidified fecal homogenates were thawed and centrifuged at 20,000 x g for 20 min; 1000 μl of supernatants was removed and added to 200 μl of internal standard (5% phosphoric acid containing 0.3% of 4-methyl-valeric acid [116.20 g/mol]). The mixture (0.2 μl) was injected onto a gas chromatograph (Bruker SCION 456-GC, Bruker Corporation, Billerica, MA, USA) and SCFAs were separated on a capillary column (Stabilwax-DA, 30 m X 0.53 mm inner diameter X 0.5 μm film thickness, Restek Corporation, Bellefonte, PA, USA) and detected with a flame ionization detector. Injector and detector temperatures were 170°C and 190°C , respectively. The column temperature was held at 90°C for 0.1 min, increased at a rate of $10^{\circ}\text{C}/\text{min}$ to 170°C , and then held for 2 min. SCFA quantification was done by calculating response factors for each SCFA relative to 4-methyl-valeric acid using the injections of pure standards. Total SCFAs were determined as the sum of acetate, propionate, and butyrate, while the relative proportion of each SCFA was determined by

$$\frac{\text{Individual SCFA}}{\text{Total SCFA}} * 100.$$

Total BCFAs were determined as the sum of isobutyrate and isovalerate.

In Vitro Assessment of Growth and Adherence

Four representative strains of human fecal origin from species known to respond to RSs, *B. adolescentis* IVS-1 (Krumbeck et al., 2018), *E. rectale* DSM 17629 (Barcenilla et al., 2000), *R. bromii* L2-63 (Ze et al., 2012), and *P. distasonis* ATCC 8503 (Sakamoto and Benno, 2006), were grown in YCFA medium as previously described (Ze et al., 2012), supplemented with a filter sterilized (0.22 μm) carbohydrate mixture (0.1% glucose, 0.1% fructose, 0.1% galactose, and 0.1% maltose; w/v). Cultures were grown at 37°C under anaerobic conditions (5% CO₂, 5% H₂, and 90% N₂). YCFA agar plates were made by adding equal volumes (1:1; v/v) of YCFA media and autoclaved 3% agar. When indicated, the carbohydrate mixture was replaced with 0.2% RS (w/v) that was 'predigested' with an *in vitro* process supposed to mimic human digestion as previously described (Jin et al., 2019).

Adherence to the RS granules was determined by methods previously described by Leitch et al., with slight modifications (Leitch et al., 2007). First, 20mg of each predigested RS were weighed into separate 1.5mL microcentrifuge tubes and sterilized using a 24 h, 70% ethanol treatment followed by a 15 min UV irradiation treatment. Overnight cultures of each strain were standardized to an OD₆₀₀ of 0.5, and then 1mL was added into the microcentrifuge tube containing the RS. After being incubated at room temperature for 15 min with agitation (350 rpm), the non-attached and loosely attached bacteria were removed prior by washing with sterile PBS (4 times), then PBS containing 0.1% Tween 80 (2 times), and then a wash with sterile PBS to remove residual Tween 80. The centrifugation time between each washing was reduced to 30 s at 700 g (Leitch et al., 2007). Strain adherence to the RS was determined by quantifying CFUs on YCFA agar plates after either 2 days (*B. adolescentis* IVS-1, *E. rectale* 17629, and *R. bromii* L2-63) or 4 days (*P. distasonis* 8503; slower growth rate on YCFA) of incubation at 37°C under anaerobic conditions. Assays were performed in triplicate.

Growth on RS substrates was determined (in triplicate) by inoculating overnight bacterial cultures (1%; v/v) into 10mL YCFA supplemented with the respective carbohydrate source, and growth was assessed through Optical density readings (OD_{600nm}) after tubes were vortexed for 10 s and then left standing for 5 min. RSs were sterilized by gamma irradiation ($\geq 10\text{kGy}$) prior to the assays. Samples were measured at intervals up to 48 h for *B. adolescentis* IVS-1, *E. rectale* 17629 and *R. bromii* L2-63, and 168 h for *P. distasonis* 8503 (slower growth rate of growth in YCFA).

QUANTIFICATION AND STATISTICAL ANALYSIS

p values, sample numbers, and names of statistical tests are provided in the main text, in the figure legends of Figures 2A–2H, 3, 4A–4E, 5A–5D, 6, S2A–B, S3A–C, and S4, as well as in Tables S2–S4, and S5. Data are reported as means \pm SD, unless described otherwise in figure legends or tables.

Statistical Analysis Software

Statistical analyses were performed using R Stats Software version 3.5.1 (R Core Team, Vienna, Austria), with ANOVAs and Spearman's rank-order correlations performed using GraphPad Prism version 8.3 (GraphPad Software, San Diego, CA, USA).

Missing Data and Outliers

When applicable, missing data were imputed by carrying the previous observation forward, assuming that no change occurred as the DF dose increased, as previously described (Streiner and Geddes, 2001). No outliers were removed from statistical analyses.

Subject Characteristics at Baseline

To determine differences between intervention groups for the assessed characteristics (e.g., age, BMI, ethnicity) at baseline, either one-way ANOVA (continuous variables) or Fisher's exact tests (count variables) were applied. Data normality of continuous variables were assessed by Shapiro-Wilk test and inspection of QQ plots. If indicated, data were square root transformed prior to statistical analysis using one-way ANOVA.

Anthropometric and Lifestyle Characteristics

Statistical significant changes from baseline to week 4 in the assessed anthropometric (weight, BMI) and lifestyle (perceived stress, physical activity, and diet) characteristics were determined by 2-way rANOVA followed by Holm-Šídák multiple comparison test to correct for multiple pairwise comparisons within each group relative to baseline. Statistical significance was considered at $p < 0.05$.

Analysis of GI Tolerability and Bowel Habits

GI tolerability and bowel movement habit data are either ordinal or derived from ordinal data (i.e., sum or mean) and consequentially are likely to be non-normally distributed. Therefore, GEE with repeated-measures models (Halekoh et al., 2006) were applied using R to assess the overall effect of treatment or dose on composite GI tolerability score and bowel movement habit data. When an overall significant effect was observed, within-group pairwise comparisons were applied using estimated marginal means (Lenth et al., 2019) followed by FDR corrections. Statistical significance was considered at FDR-adjusted q values < 0.05 . Individual GI symptom data were further analyzed using cumulative link models (Christensen, 2018) to individually assess the effect of treatment and dose, where $p < 0.05$ was considered statistically significant.

Analysis of Fecal SCFAs

To determine the statistical significance of within-group changes at each treatment dose (i.e., 10 g/d to 50 g/d) relative to baseline (0 g/d), individual and total SCFA and BCFA concentrations, SCFA proportions, and BCFA to SCFA ratio were analyzed using 2-way rANOVA followed by Holm-Šídák multiple comparison test. To determine whether the overall changes in fecal SCFAs induced by the four treatments were significantly different between each intervention group, acetate, propionate, and butyrate concentrations and relative proportions, as well as BCFA to SCFA ratio data were analyzed using ordinary 2-way ANOVA followed by Holm-Šídák multiple comparison test. For these analyses, all four delta values from each treatment dose (i.e., $\Delta 10\text{-}0\text{g}$ to $\Delta 50\text{-}0\text{g}$) and for each subject were treated equally as replicates. Application of this statistical approach therefore assessed differences in the overall change in SCFAs during the intervention without consideration of supplementation dose, with an assumption that normal fluctuations over

time would report a mean change near zero; while consistent, dose-dependent changes would report a clear mean positive or negative change. Further, even though ANOVA are considered to be a robust statistical approach for the analysis of data that may violate the general assumption of normal distribution (Harwell et al., 1992; Schmieder et al., 2010), normality of ANOVA residuals were assessed using Shapiro-Wilk test and inspection of QQ plots. If the residuals were not normally distributed, square root transformations of SCFA or BCFA data were done prior to statistical analysis with ANOVA. Statistical significance was considered at $p < 0.05$.

Analysis of Bacterial Community Composition

To determine the statistical significance of the changes observed in bacterial β - and α -diversity metrics, both temporal within-treatment group (effect of dose relative to baseline) and between-treatment groups (differences of shifts induced by RS4s and placebo at each dose), 2-way rANOVA were applied followed by Holm-Šídák multiple comparison tests. If the residuals were not normally distributed, square root transformations of diversity metrics data were done prior to statistical analysis with ANOVA. Non-metric multidimensional scaling plots, PERMANOVA, and multivariate dispersion analyses (PERMDISP) were performed using the metaMDS, Adonis, and betadisper functions, respectively, from the vegan package in R (Oksanen et al., 2017) to determine the statistical significance of RS4-induced changes in inter-subject β -diversity when compared to baseline and to the analogous changes by placebo. Statistical significance was considered at $p < 0.05$.

Furthermore, statistical significance of the changes observed in relative abundance of bacterial taxa and CARGs, both temporal within-treatment group (effect of dose relative to baseline) and between-treatment groups (differences of shifts induced by the RS4s relative to placebo), was determined by 2-way rANOVA and ordinary 2-way ANOVA (where the 4 delta values for each dose were treated equally as replicates, i.e., $\Delta 10-0g$ to $\Delta 50-0g$), respectively. To control for multiple comparisons, FDR corrections were applied to p values using Prism, whereby statistical significance was considered at FDR-adjusted q values < 0.05 .

Fecal Microbiome and SCFA Correlations

Dose-response relationships were evaluated using Spearman's correlations to assess monotonic relationships between treatment doses (i.e., 0 g/d to 50 g/d) and fecal microbial abundance and SCFA concentrations. Statistical significance was considered at $p < 0.05$. Spearman's correlations were further applied to assess correlations between changes in fecal microbial abundance and the relative proportion of fecal SCFAs. To account for the extensive comparisons made, FDR corrections were applied to p values using Prism, whereby statistical significance was considered at FDR-adjusted q values < 0.05 .

DATA AND CODE AVAILABILITY

The 16S rRNA sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive. The accession number for the sequencing data reported in this paper is BioProject: PRJNA560950.