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Effects of Resistant Starch Type III Polymorphs on Human Colon Microbiota and Short Chain Fatty Acids in Human Gut Models

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This study probed the possible effects of type III resistant starch (RS) crystalline polymorphism on RS fermentability by human gut microbiota and the short chain fatty acids production in vitro. Human fecal pH-controlled batch cultures showed RS induces an ecological shift in the colonic microbiota with polymorph B inducing *Bifidobacterium* spp. and polymorph A inducing *Atopobium* spp. Interestingly, polymorph B also induced higher butyrate production to levels of 0.79 mM. In addition, human gut simulation demonstrated that polymorph B promotes the growth of bifidobacteria in the proximal part of the colon and double their relative proportion in the microbiota in the distal colon. These findings suggest that RS polymorph B may promote large bowel health. While the findings are limited by study constraints, they do raise the possibility of using different thermal processing to delineate differences in the prebiotic capabilities of RS, especially its butyrogenicity in the human colon.

KEYWORDS: Resistant starch; prebiotics; butyrate; bifidobacteria; human colon microbiota

INTRODUCTION

Modern Western style diets and more recent sociological changes such as a more sedentary lifestyle are increasingly recognized as important contributors to the observed increase in obesity and chronic diseases such as coronary vascular disease and some cancers (*1*). Two current approaches try to tackle these rising problems, one is dietary advice and guidelines issued by health authorities and the other is the use of food supplements, functional foods, and nutraceuticals. Starch is a commonly consumed substance found in various foods that undergo thermal processing as an integral part of human staple diets making up to over 50% of the daily energy intake in agrarian cultures and some 25% of westernized societies (*2*). It is now known that some of the ingested starch escapes digestion by the mammalian digestive enzymes in the small intestine and reaches the large bowel where it may be fermented by the colon microbiota (*3*). This nondigestible or "resistant" starch (RS) is classified into four types (*4*), with type III being the result of pasted starch formed by thermal processing in the presence of water followed by retrogradation (*5*). Some of the current uses of RS include modification of food texture, use as a crisping agent, and recently attention has been drawn to its ability to affect the gut microbiota and aid in the prevention of cancer tumor development (*3, 6, 7*).

Consumption of RS has been linked to a number of potential health benefits including a prebiotic type fermentation within the colon, increasing bile acid turnover, and lowering secondary bile acid formation in rats and human-microbiota associated rats (*6, 8, 9*). RS_{III} has also been shown to increase butyrate concentrations in the feces of human volunteers (*10*), a finding of apparent importance, since butyrate has been shown to be important for normal epithelial cell growth and prevention of colonic disease (*11, 12*). Furthermore, a recent review (*3*) describes various studies that have shown RS to have benefical effects ranging from hypoglycaemic effects to reducing the risk of certain intestinal diseases.

Starch in plants appears as granules and can be fractionated into two glucose homopolysaccharide macromolecules: amylose and amylopectin. For most native starches, X-ray diffraction (XRD) yields two types of spectral patterns, A type and B type (*13*). Generally, heat treatment processing of starch increases the RS content in foods (*14–17*). Upon heating, the crystalline structures within the starch granule lose order, commonly termed as starch melting, preceding the formation of a gel network made of amylose and amylopectin readopting helical structures. The increase in crystallinity, referred to as retrogradation, takes several hours (in high amylose starch) to several days (in high amylopectin starches) and is affected by parameters such as a decrease in branching (*18*) or repeated heating and cooling cycles, which increase retrogradation (*19, 20*). The formation of RS_{III} during thermal processing and its structural properties are also affected by the amylose/amylopectin ratio, chain length,

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retrogradation conditions, annealing, lipids, and solution composition (*15, 21, 22*). In most cases, retrograded starch forms a B-type pattern (*13*); however, an A-type structure can be detected when the starch is held for extended periods at high temperature after gelatinization (*5, 15*). Recently, it was shown that retrogradation of starches, including high amylose corn starch (HACS), at 40 °C or at 95 °C leads to the formation of a B-type polymorph or a mixture of A- and V-type polymorphs, termed A polymorph (*23*). Although these polymorphs were found to have similar melting temperatures ($T_m \cong 140-170$ °C), they were also found to differ in their crystalline structure as inferred from powder X-ray diffraction and small angle X-ray diffraction (*23, 24*).

Interestingly, this polymorphism of RS_{III} was found to affect the bacterial fermentations of batch cultures based on rat feces (*25*). Thus, we sought to probe the possible effects of RS crystalline polymorphism formed by the thermal processing of HACS on the human colonic microbiota, using a validated in vitro model of the human colonic microbiota fed with chemostat media. By that, we hoped to truly link bacterial growth with the fermentation of the test carbohydrate, which was the sole carbohydrate in the media.

MATERIALS AND METHODS

Materials. *Carbohydrates.* Four carbohydrates were used in these experiments, HACS (Hylon VII, National Starch, NJ) used to produce the resistant starch (RS) samples, fructooligosaccharides (Oligofructose OF, Orafti, Belgium), and two RS polymorphs, termed A and B.

Enzymes and Chemicals. Amyloglucosidase (A3042, 11 500 units·mL^{-1}), heat stable α-amylase (A4551, 520 000 units·g^{-1}), and protease (P6911, 4600 units·g^{-1}) were purchased from Sigma Co. (Israel) and used as received; all other reagents were of analytical grade.

Bacterial Growth Media. All culture experiments were conducted using sterile Chemostat basal media containing the following: 2 g/L peptone water, 2 g/L yeast extract, 2 g/L $NaHCO_3$, 0.5 g/L bile salts (No. 3), 0.5 g/L cysteine·HCl, 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.01 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 6H_2O$, and 0.005 g/L Hemin. Additionally, 2 mL of Tween80, 10 μL of Vitamin K1, and 4 mL of Resazurin solution (0.025% w/w) were added to every 1 L of media.

Thermal Production of RS Polymorphs. For the scale of production needed for these experiments, RS was produced via a modified version of the protocol previously described (*23, 24*). Basically, the procedure contained two steps: First, HACS was thermally processed in order to produce RS_{III}, and then the RS was isolated from the sample using enzymatic digestion of the non-RS fraction. In these experiments, we autoclaved 1 L of 10% HACS solutions (w/w) at 121 °C for 120 min; after that, samples were allowed to retrograde for 24 h at different temperatures: 40 °C for the production of RS_{III} polymorph B (RS_B) and 95 °C for RS_{III} polymorph A (RS_A). Immediately after retrogradation, samples were meshed, filtered, and dispersed in 5 L of phosphate buffer (pH = 6) containing 0.004 g/L of heat stable α-amylase (~2000 units/L). After digestion for a period of 30 min at 90 °C, samples were cooled on ice to room temperature. Then, the pH was brought to 4.5 using 2% phosphoric acid, the temperature was brought up to 60 °C, and 1 mL of amyloglucosidase (final activity of ~2300 units/L) was added for a period of 2 h. The resulting slurry solution was then centrifuged at 3000 rpm for 16 min (at 10 °C). The pellet was then dissolved in 5 L of phosphate buffer (pH = 7.5) and treated overnight with 100 mL of protease solution (0.016 g of protease dissolved in 100 mL of phosphate buffer, pH = 7.5). The remaining starch fraction, corresponding to RS_{III}, was then separated by centrifugation at 10 °C, 3000 rpm for 16 min, with two cycles of pellet washings with double distilled water. Before storage and further analysis, the pellet was freeze-dried and pulverized. Polymorph formation was then verified by the X-ray diffraction pattern obtained by a Philips PW 3020 powder diffractometer equipped with a graphite crystal monochromator. The operating conditions were as follows: Cu $K\alpha_1$ radiation (0.154 nm), voltage (40 kV), and current (40 mA). Samples were scanned over the range 5−30° 2θ in steps of 0.02° 2θ per 4 s, and the crystalline nature of the complex was determined by the position of the peaks as compared to earlier work (*23, 26*).

Human Microbiota Fermentation of RS Samples. *pH Controlled Fecal Batch Cultures.* Fermentation of RS_{III} polymorphs was studied in triplicate pH controlled batch cultures inoculated with human feces from three healthy adult volunteers. Each batch culture comprised 180 mL of fermentation medium (described above) and the test carbohydrate (1% w/v) and 20 mL of a 10% (w/v) fecal slurry prepared with prereduced phosphate buffered saline (0.1mol/L, pH 7.0, Sigma, U.K.). Anaerobic conditions were maintained by continuous sparging of the fermentation with O_2-free N_2. The temperature was kept at 37 °C using a water jacket, and pH values between 6.5 and 6.8 were maintained by the addition of 0.5 M NaOH or HCl. Fecal batch cultures were done in triplicate and conducted over a period of 24 h, with samples collected aseptically at 0, 5, 10, and 24 h (*27, 28*).

Three Stage Continuous Culture System. Simulation of the different microbiological characteristics of the proximal, transverse, and distal colons was achieved through a three stage continuous system inoculated with an adult human feces sample, as previously described and validated (*29*). The system comprised three serially connected fermenter vessels continuously fed with culture medium containing 1% (w/v) of the test carbohydrate. This medium was pumped into vessel 1 (V1) with operating volume of 80 mL, which sequentially fed Vessel 2 (V2) then Vessel 3 (V3), both with an operating volume of a 100 mL, and finally collected as waste. The average retention time was measured and maintained at an average 37.3 h, which in a physiological context would comply with a transit time of 37.3 h. Each vessel was magnetically stirred and maintained under anaerobic conditions by continuous sparging with O_2-free N_2. Temperature and pH were automatically controlled so that each vessel was kept at 37 °C and pH values of 5.5, 6.2, and 7.1 in V1, V2, and V3, respectively. Samples were collected 24 h after inoculation ($t = 0$) and at steady state after 9, 10, and 11 days. All experiments used chemostat medium described above, in which the only carbohydrates present were the individual test carbohydrates, RS_A, RS_B, or native HACS as control.

Microbial Enumeration and Quantification of SCFA Content. *Microbial Enumeration Using FISH.* Duplicate samples collected from the gut model and batch culture vessels were fixed with 4% paraformaldhyde at 4 °C overnight, centrifuged for 5 min at 13 000 rpm, washed with PBS (0.1mol/L, pH = 7.0, Sigma, U.K.) solution, and centrifuged twice for 5 min at 13 000 rpm at a time. Then, the resulting pellet was resuspended in PBS/ethanol (1:1 v/v) and kept at −20 °C before further processing. Frozen samples were then thawed and diluted in a hybridization buffer (1.36 M NaCl, 30 mM Tris−HCl, pH = 7.2) with 0.15% SDS, prewarmed to hybridization temperature. The cells were then hybridized with one of four 16S rRNA fluorescent probes for *Bacteroides* (Bac 303) (*30*), *Bifidobacterium* (Bif 164) (*31*), *Eubacterium rectalae* (Erec 482) (*32*), and *Atopobium* group (Ato 291) (*33*), according to conditions specified in the literature. For the enumeration of total cells, samples were stained with the nucleic acid stain 4′,6′-diamino-2-phenylindole (DAPI). Slides were enumerated using a Nikon microscope with an EPI-fluorescence attachment (Nikon U.K., Kingston-upon-Thames, U.K.) (*34*).

Determination of Short Chain Fatty Acid Content. Analysis of the short chain fatty acid (SCFA) content in samples collected during the batch cultures was determined by gas chromatography with proper internal standards; for example, for butyrate determination, ethyl butyrate served as the internal standard. Batch culture fermentation sample (1 mL) was centrifuged at 13 000 rpm for 10 min. The supernatant was filtered through a 0.2 μm filter into a 1.5 mL eppendorf tube for storage at 4 °C until use. A portion of the supernatant (100 μL) was added to 400 μL of ethyl butyrate in GC glass vials and then capped. Samples of 1 μL were injected into a GC (HP 5890 A Hewlett-Packard GmbH) equipped with a flame ionization detector and a capillary column (25 m × 0.23 mm) impregnated with 20 m Carbowax (Hewlett-Packard GmbH). The carrier gas was He at a column flow rate of 12 mL/min with a split ratio of 1:12, and the column temperature was 125 °C.

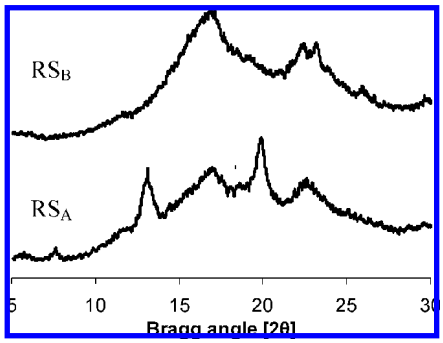

Figure 1. X-ray diffraction patterns of resistant starch type III polymorphs produced from HACS, values expressed as arbitrary intensity units at the corresponding Bragg angles.

Statistical Analyses. The statistical significance of the bacterial counts in the batch cultures and gut models was tested using analysis of variance (one way ANOVA) and *t* test analyses (assuming equal variances) to test the specific bacterial counts and the total bacterial counts. Instances of batch cultures in which a statistical difference ($p < 0.05$ or better) was found were also further tested using a *t* test (assuming equal variances) to evaluate differences between every two experiments (i.e., comparison between results achieved with RS_A versus results achieved using RS_B). In light of the study hypothesis, only the statistically significant differences between RS_A and RS_B fermentations are described in the results section. Additionally, *t* test (assuming equal variances) was implemented to evaluate the statistical significance of the SCFA levels determined for the fermentations of RS_A and RS_B. All of the statistical analyses were conducted using Microsoft Office Excel software for Windows XP.

RESULTS

Thermal Production of RS Polymorphs. Experiments conducted using the modified method of RS production from HACS proved to successfully enable an average weight yield of about 8% for RS_A and a slightly higher yield of about 11% for RS_B (data not shown). As can be seen in **Figure 1**, the resulting RS powders showed two distinct X-ray diffractions attributed to B polymorph and a polymorph mixture previously referred to as A polymorph (*23*).

Human Gut Microbiota Fermentations of RS Polymorphs. *Batch Cultures.* Powders of both RS_A and RS_B, previously tested and controlled for purity, were used as the sole carbohydrates in the culture medium used for the anaerobic fermentations of diluted fecal samples, with HACS and FOS as control carbohydrates. Fermentation experiments conducted over a period of 24 h enabled bacterial and biochemical analysis. FISH enumeration of the total bacteria in the samples, depicted in **Figure 2**, showed no significant differences in the total counts during the fermentation of either RS_A or RS_B. It was also noted that, during the first hours of fermentation, total counts slightly declined but regained initial counts after 24 h. In the fermentations of RS_A, RS_B, and FOS, the decrease in bacterial counts was observed in the first 10 h of fermentation, while in the fermentation of native HACS, total counts began to rise after 5 h. This retardation period is believed to arise from the use of chemostat media with only the pure test carbohydrates added as a single carbon source. This experimental design aimed to help detect the bacterial species fermenting the test carbohydrate, which were expected to be the only species who can increase in numbers. Accordingly, the fact that, in the fermentation of native HACS, total colon bacteria decline in numbers only during the first 5 h of fermentation suggests a shorter time needed for bacteria to adjust to the fermentation of native HACS compared to the other carbohydrates. This suggests that RS_A, RS_B, and FOS have similar accessibility to fermentation by adult human microbiota and that their fermentation is slower than that of native HACS.

Interestingly, analyses of specific major fermentative bacterial populations in the microbiota, shown in **Figure 3**, indicate both RS polymorphs supported the growth of different profiles of bacteria. Throughout the fermentation, RS_A supported higher numbers of the *Bacteroides* species than all other carbohydrates. Statistical analyses of the growth profiles elucidated a few differences between the fermentations of the carbohydrates. For the bifidobacteria, **Figure 3**A depicts that RS_B batch cultures showed significantly ($p < 0.01$) higher counts after 10 h compared to RS_A, with a growth profile similar to that of the prebiotic FOS. Indeed, batch cultures maintained on RS_A showed the lowest bifidobacterial counts at 24 h compared to the other test carbohydrates. Additionally, native HACS was found to yield similar bifidobacterial levels as RS_B and FOS. *Bacteroides* spp. counts described in **Figure 3**B showed that, after the first 5 h, FOS and RS_A supported similar bacterial counts, however, in batch cultures maintained on RS_A, *Bacteroides* spp. counts were significantly ($p < 0.001$) higher than in FOS batch cultures after 10 h. In the case of *Atopobium*, **Figure 3**C shows the differences that were noted after 5 and 10 h. This figure shows that FOS yielded the highest counts and RS_A yielded significantly ($p < 0.01$) higher counts than RS_B. In this respect, RS_B yielded counts similar to those reached in the fermentation of the native HACS, while RS_A yielded elevated *Atopobium* counts similar to those reached in the fermentation of FOS, with final counts (at 24 h) being practically the same. For the *Eubacterium rectalae* group, **Figure 3**D shows that no significant differences between RS_A, RS_B, and FOS were observed throughout the fermentation. However, a slight decrease in bacterial counts is observed after 5 h when RS_A or native HACS are the sole carbohydrates.

Three Stage Continuous Culture System. The impact of native HACS, RS_A, and RS_B on the microbial ecology within different physiological regions of the colon was investigated using a validated three stage continuous model of the human colonic microbiota. Results, given in **Figure 4**, show that, at approximated steady-state conditions (reached after 9−11 days), all carbohydrates yielded different bacterial profiles, especially when comparing the RS_A-fed system to the RS_B-fed system. This comparison showed that bacterial counts differ both within the different vessels of the gut model and between the different gut models.

Comparison between the Effects of RS_A and RS_B. *t* Test (assuming equal variances) statistical analyses were also done to compare bacterial counts reached in the two systems fed with the different RS polymorphs. These statistical tests, denoted in **Figure 4**, gave rise to two major observations. One was that RS_B supported significantly lower total bacterial numbers ($p < 0.01$) in the proximal and distal parts of the colon, even when compared to the native HACS. The other was that RS_B enabled bifidobacteria to reach significantly ($p < 0.01$) higher counts in the proximal colon compared to the counts reached when the system was fed with RS_A. Additionally, in the RS_B-fed system, the proportion of *bifidobacteria* and *Atopobium* species out of the total bacteria in the distal colon was double that of the RS_A-fed system or control system fed with native HACS.

A control experiment done with native HACS showed no significant inner differences between all groups of bacteria in the vessels simulating the different parts of the colon. However, when compared to the RS-fed systems, it was found that,

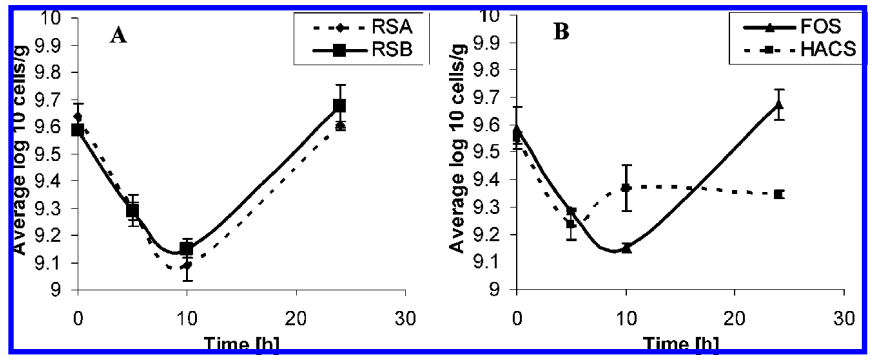

Figure 2. Population levels of total bacteria enumerated in fecal batch cultures using nucleic acid stain 4′,6′-diamino-2-phenylindole (DAPI): (**A**) Cultures maintained on media containing RS polymorph A (RS_A) or polymorph B (RS_B), as the sole carbohydrate; (**B**) Cultures maintained on media containing HACS or FOS, as the sole carbohydrate. Bacterial numbers are expressed as $\log_{10}$ cells/mL batch culture (mean ± SD, $n = 3$), with no significant differences between cultures throughout the fermentation.

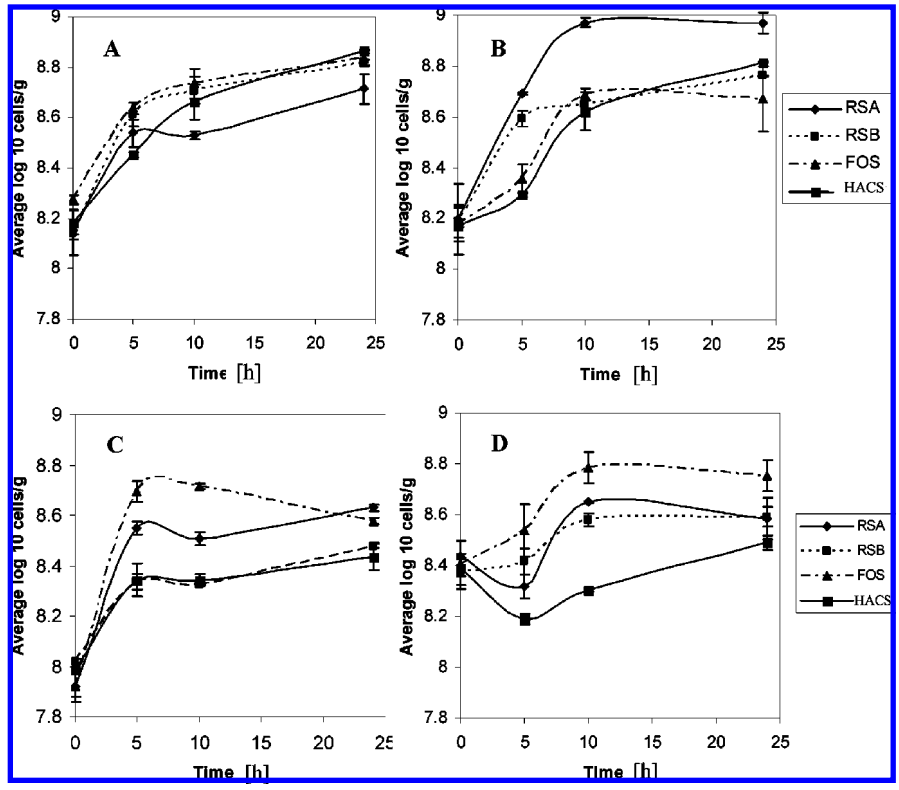

Figure 3. Population levels of *Bifidobacteria* (**A**), *Bacteroides* (**B**), *Atopobium* (**C**), and the *Eubacterium rectalae* group (**D**) in fecal batch cultures using fluorescent in situ hybridization and 16S rRNA targeted probes. The batch cultures of human feces were maintained on chemostat media containing fructooligosaccharide (FOS), native HACS, RS polymorph A (RS_A), or polymorph B (RS_B), as sole carbohydrate. Bacterial numbers are expressed as $\log_{10}$ cells/mL batch culture, (mean ± SD, $n = 3$).

regardless of its polymorphism, RS enabled the *Eubacterium rectalae* group to reach higher counts in the lower parts of the colon (i.e., the transverse and distal colon).

Short Chain Fatty Acid Production. Short chain fatty acids are part of the products of bacterial carbohydrate metabolism. Because of their health related implications on colon health and prevention of disease, the production of acetic, propionic, and buyric acid was monitored throughout the batch culturing of the human fecal samples, and the relevant concentrations determined by GC are given in **Table 1**. These analyses show that acetate is produced throughout the fermentations, while propionate and butyrate start to appear in detectable concentrations after 10 or 24 h, respectively. When comparing the concentrations of acetate produced in the fermentations of RS_A or RS_B, no significant differences were found. However, production of propionate and butyrate appear to be affected by the resistant starch polymorphism. According to the findings described in **Table 1**, RS_B appears to induce higher levels of propionate and butyrate production compared to RS_A. Moreover, it was found that cultures grown on RS_B yielded significantly ($p < 0.01$) higher butyrate concentrations than cultures grown on RS_A.

DISCUSSION

The study provides preliminary results of the possibility of using differences in crystalline polymorphism of resistant starch (RS) type III (formed by thermal processing of HACS) to affect its fermentability by human gut microbiota. These different RS_{III} polymorphs were produced through a simple method in which HACS was autoclaved and then allowed to retrograde in an oven at either 40 or 95 °C for RS_B and RS_A formation, followed by

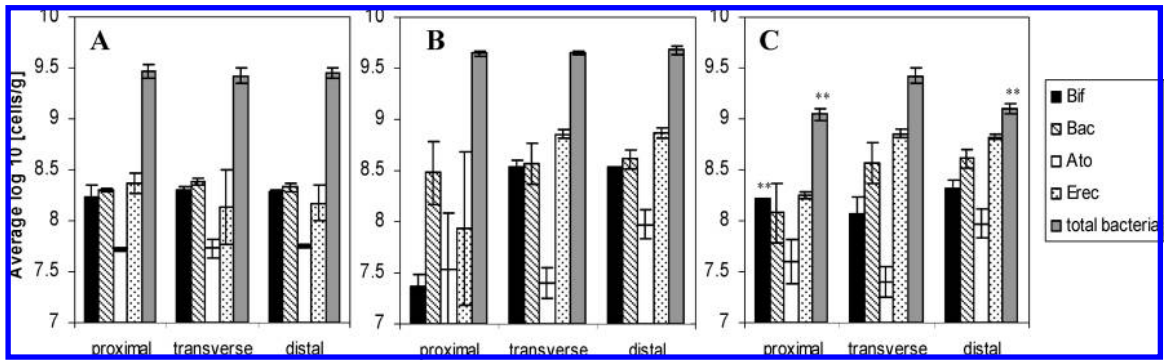

Figure 4. Mean *Bifidobacteria*, *Bacteroides*, *Atopobium*, *Eubacterium rectalae*, and total bacteria bacterial counts enumerated by FISH in continuous human gut model systems fed for 11 days with native HACS (**A**), RS_A (**B**), or RS_B (**C**), as sole carbohydrates in the growth media. Values are expressed as $\log_{10}$ mean cells/mL ± SD, as determined by duplicate samples collected after 9, 10, and 11 days of fermentation. $^{**}p < 0.01$ (*t* test comparison of RS_A vs RS_B).

Table 1. Average Concentrations (mM) of Short Chain Fatty Acids Measured during 24 h Human Feces Fermentation (pH Controlled Batch Cultures) of RS Polymorph A (RS_A) or B (RS_B) Expressed as Mean ± SD ($n = 3$).

	acetic acid (mM)		propionic acid (mM)		butyric acid (mM)	
time (h)	RS_A	RS_B	RS_A	RS_B	RS_A	RS_B
0	0.51 ± 0.72	not detected	not detected	not detected	not detected	not detected
5	3.67 ± 1.17	2.91 ± 1.56	not detected	not detected	not detected	not detected
10	7.32 ± 5.27	8.43 ± 1.62	0.67 ± 0.94	0.52 ± 0.66	not detected	not detected
24	17.14 ± 6.41	14.67 ± 0.73	4.03 ± 0.42	5.29 ± 0.45	0.14 ± 0.10	0.75 ± 0.25[a]

[a] $p < 0.05$ significance of statistical difference between RS_A and RS_B.

an enzymatic digestion of the non-RS fraction and lyophilization. The resulting RS powders showed two distinct X-ray diffractions, as shown in **Figure 1**. These diffractions, which correspond with previous work studying RS polymorphism (*23, 24*), are attributed to B polymorph and a polymorph mixture referred to as A polymorph.

Twenty-four hour fecal batch cultures, maintained at pH 6.5, showed that RS was slowly fermented by the human colonic microbiota, with **Figure 2** showing total counts decreasing (by over 0.5 log) in the first 10 h of fermentation but regaining initial counts after 24 h. Simultaneously, **Figure 3**A, **B**, and **C** shows an increase in *Bacteroides*, *Bifidobacteria* and *Atopobium*, suggesting the test carbohydrates (RS_A and RS_B) induced an ecological shift. It is also hard to ignore the growth of *Bifidobacteria* found to be induced by RS_B and the growth of *Atopobium* induced by RS_A. The results obtained in this study are also supported by a previous study done in rats and human microbiota-associated rats, which demonstrated RS_{III} to promote the growth of lactobacilli and bifidobacteria along with a concomitant decrease in enterobacteria (*6*). Here, it is shown that RS_{III} polymorph B appears to support the growth of bifidobacteria in in vitro models of the human colonic microbiota while maintaining total counts, probably due to a decrease in other bacterial species or groups.

Comparison of the changes in the bacterial counts measured during batch culture growth on the RS polymorphs showed that RS polymorphism enabled varying growth of *Bacteroides* spp., *Bifidobacterium* spp., and *Atopobium* spp. with no significant differences in the growth of the *Eubacterium rectalae* group. Specifically, RS_B exerted a higher bifidogenic effect than RS_A, while RS_A fermentation in pH controlled batch cultures supported higher numbers of *Bacteroides* and *Atopobium* than RS_B after 24 h. Interestingly, to the best of our knowledge, this is the first time RS_{III} has been reported to induce *Atopobium* growth, with RS_A stimulating *Atopobium* growth better than RS_B and much like the prebiotic FOS. Thus, it seems that the B polymorph is more accessible to *Bifidobobacterium* species while the A polymorph mixture is more readily fermented by the *Bacteriodes* and *Atopobium*. In this context, it might also be concluded that the *Eubacterium rectalae* group cannot ferment either of the RS polymorphs.

As in previous studies of in vitro fermentations by human gut microbiota, this study also tested RS effects on colonic bacterial ecology in a three stage continuous fermentation system simulating the proximal, transverse, and distal parts of the human colon (*27, 29*). Differences in the bacterial composition of the colonic microbiota by loci within each model were expected because of the different conditions present in each part of the colon. However, as far as we know, this is the first experimental report showing that RS_{III} polymorphs, originating from the same source and produced similarly, exert different effects on the human colonic microbiota. Furthermore, comparison between the microbiotal compositions reached in the RS-fed in vitro gut models illustrates different selectivity of the two polymorphs and supports the findings found in the batch cultures. Thus, RS_B proved to help maintain higher bifidobacteria in the proximal colon along with lowered total counts, suggesting selective enrichment of bifidobacteria's fraction in the flora. On the other hand, RS_A better supported the growth of the *Atopobium* group in the distal colon. However, due to the limited nature of the experiments, these findings should be further tested in animal or human feeding studies.

As the most common site for colonic cancer tumors is the distal colon, studies have attempted to induce fermentation of complex carbohydrates in the lower parts of the colon, mainly in the distal colon. One example is a study where a RS−psyllium mixture was used to enhance RS fermentation in the distal colon of rats (*35*). Previous work has also demonstrated that starch hydrolysates commonly used in the food industry (i.e., dextrins and maltodextrins) are mainly broken down in the proximal and transverse colon; however, this study lacked reference to the crystalline form of the polysaccharides (*27*). Bacterial growth profiles noted in the batch cultures described here seem to indicate RS_{III} is slowly fermented in the human colon. Ad-

ditionally, **Figure 4** illustrates that the spatial effects of the RS_{III} polymorphs tested here seem to indicate that RS_A helps maintain total bacterial counts throughout the colon better than RS_B while RS_B enriches the fraction of bifidobacteria in the proximal colon. Thus, it would appear that the prebiotic selectivity of the RS polymorphs may be altered by different thermal processing of the raw native HACS.

Production of short chain fatty acids, mainly of butyrate, has been deemed a favorable trait of colon-targeted functional foods due to their proven beneficial physiological effects on the growth and differentiation of colonic epithelial cells (*11, 36–38*). Recently it was demonstrated that RS_{III} and its polymorphs induce butyrate production (*10, 11, 25*). The formation of butyrate from butyryl CoA as a bacterial metabolite is considered to arise from two possible pathways, one by butyrate kinase and another by butyryl CoA/acetate CoA mediated by free acetate (*39*). Major butyrogenic species within the human colonic microbiota have been identified in the genera *Clostridium*, *Eubacterium*, and *Fusobacterium* (or now *Faecalibacterium*) (*33, 39, 40*). Incubations of human fecal samples suggest that free acetate significantly contributes to the carbons found in butyrate produced by the innocula (*41*). The data obtained in the before mentioned batch cultures showed a 5 and 10 h lag in the production of propionate and butyrate, respectively, while acetate is continuously produced in increasing amounts throughout the fermentation. These observations may suggest that RS_{III}-induced butyrate production is formed via butyryl CoA/acetate CoA upon the accumulation of sufficient acetate in the medium. Thus, one might speculate that butyrate is formed as a secondary metabolite indicative of a metabolic shift induced exogenously by acetate; however, the present study cannot confirm or refute which is the true cause of butyrate production. **Table 1** portrays an interesting preliminary result, which needs further confirmation, in this respect. According to **Table 1**, RS_B induces significantly higher butyrate production than RS_A, leading to the production of almost 1 mM butyrate, a finding which might be significant, since levels of 1−10 mM butyrate have in vitro effects on human cells (*39*). This raises the possibility that butyrate levels formed during RS_B fermentation might exert some beneficial effects.

Overall, the findings appear to suggest that different thermal processing of HACS leading to the formation of different crystalline polymorphs of RS_{III} may have differential fermentation profiles within the gut microbiota. RS_A appears to induce the growth of *Atopobium* much like FOS, while RS_B appears to induce bifidobacteria growth along with elevated butyrate production, indicating that the two polymorphs of RS may be handled differently by the gut microbiota. Although the findings described here are limited by the preliminary nature of the study, one cannot ignore the exciting possibility of using thermal processing to delineate differences in the prebiotic capabilities of RS_{III}. Future studies in humans are required to confirm the prebiotic nature of resistant starch type III and its different polymorphs.

ABBREVIATIONS USED

FOS, Fructooligosaccharide; GIT, gastrointestinal tract; HACS, high amylose corn starch; PBS, phosphate buffer saline; RS, resistant starch; RS_{III}, resistant starch type III; RS_A, resistant starch type III polymorph A; RS_B, resistant starch type III polymorph B; SCFA, short chain fatty acids; XRD, X-ray diffraction.

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