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Novel resistant starch type 4 products of different starch origins, production methods, and amounts are not equally fermented when fed to Sprague-Dawley rats

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Abstract

Scope—The possible mechanisms of production of four novel RS4 products for total cecal fermentation in an *in vivo* rodent model were evaluated.

Methods and Results—Forty weanling rats were randomly assigned to five groups (n=8) for a 3-week study. Starches were the RS type 4 products as 10% of weight of RS diets (RSA-RSD), and AMIOCA® starch (100% amylopectin) comprised of 53.6% weight of control (CON) and 43.6% weight of RS diets. The RS products varied by percent purity and origin (potato, corn, tapioca). At euthanasia, cecal contents, serum, GI tract, and abdominal fat were collected. Emboweled body weight (EBW) was calculated as body weight minus GI contents. RSB, RSC, and RSD fed rats had greater empty cecum weights, lower cecal contents pH, higher cecal contents wet weight, higher total cecal contents acetate and propionate than the CON and RSA fed rats. Two other indicators of fermentation, total cecal contents butyrate and GLP-1, did not have significant ANOVA F values, which required more subjects for 80% power.

Conclusions—RS4 products that are produced from different starch origins with varying amounts of RS4 content and different methods of production are not uniformly fermented in an *in vivo* model.

Graphical Abstract

Fermentation of dietary resistant starch has been associated with positive gut health benefits. Rats were fed customized diets using one of four unknown resistant starch type 4 products. Three of the four products appeared to be readily fermented by the rats. Fermentation of the product by an animal model can assess the potential benefits of these products as a functional food.

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Author contributions: DC is faculty and laboratory manager for *in vivo* animal studies at LSU AgCenter, RP and JG were graduate students assisting rat study, AMR performed laboratory analyses, BM is a statistician and faculty, VG and MS are employees at Ingredion Incorporated, MJK is PI, mentor, and assisted in design of study.

Keywords

Fermentation; Glucagon-like peptide 1; Resistant Starch; Resistant Starch Type 4; Short-Chain Fatty Acids

1 Introduction

Resistant starch (RS) was first described by Englyst et. al. as “starch and starch-degradation products” which “reach the human large intestine and thus become available to microbial fermentation”.^[1] These starches are also known as fermentable fibers that largely bypass digestion in the small intestines and serve as prebiotic “food” for the large intestine bacteria.^[2] Resistant starch is appealing for application in the food industry not only as a natural, functional food source with whole body health benefits, but also because it imparts a better appearance, texture, and mouthfeel than other traditionally used fibers.^[3] There are four types of resistant starch conventionally recognized. RS1, RS2, and RS3 are naturally found in foods such as whole grains, legumes, raw potatoes, and cornstarch.^[2] RS1 is physically inaccessible and bound in a cell wall matrix that prevents digestion; RS2 is ungelatinized amylose starch granules with a complex crystalline structure. RS3 is nongranular, “retrograded amylose” found in foods that are cooked and then cooled such as potatoes and puddings. RS4 is chemically-modified starch generated by several different methods including the addition of ester crosslinks between starch molecules, the addition of chemical constituent groups, or by acid hydrolysis and heat treatment.^[4–10] These methods alter the chemical and physical properties of the starch which reduce its digestibility by preventing enzymatic accessibility and/or degradation.^[8–10] In the current study, three of the RS4 starches were distarch phosphates with cross-linking^[7, 8], and one was produced by acid hydrolysis and heat treatment^[9]. Finally, a possible fifth type RS5, comprised of amylose-lipid complexes prevents amylase enzymes from digesting the starch.^[10]

Our research group has studied RS as a functional food and has found important metabolic health benefits when fermented, which include reducing abdominal fat with isocaloric diets, improving insulin sensitivity, and increasing serum GLP-1, a satiety hormone.^[5, 11, 12] In 2018, FDA included RS, specifically RS2, as a dietary source of fiber with sound physiological benefits for human health such as lowering post-prandial insulin levels.^[13] The improved health parameters indicate promising prebiotic/fiber therapeutic approaches for humans. The challenge is that some people respond favorably to probiotic and prebiotic interventions while others are termed “non-responders” and do not appear to gain the health benefits from RS fermentation.^[14]

Fermentation of RS results in the production of SCFA, in particular acetate, propionate, and butyrate, which lowers the intestinal pH, and can modify the gut bacterial composition.^[15] Butyrate is considered an important energy source for colonic epithelium.^[16] It is beneficial for colon health by reducing inflammation and allergic responses in the gastrointestinal tract as well as strengthening the gut barrier, suppressing colonization of enteric pathogens, and preventing certain cancers such as colorectal cancer.^[15, 17] Acetate, especially, and propionate are produced in much greater amounts during colonic fermentation of RS

compared to butyrate. [16] Both have been associated with a reduction in food intake and an increase in GLP-1 by stimulating the free-fatty acid receptors, FFAR2 and FFAR3 (also called GPR43 and GPR41, respectively), found in adipose tissue, the gut, and the peripheral nervous system thus affecting the gut-brain axis signaling pathways. [18,19] Propionate and butyrate are suggested to be an integral player in the proposed mechanisms of intestinal gluconeogenesis and a critical regulator of energy homeostasis resulting in decreased body weight and adiposity.[19]

Our research group has found that RS2, a high-amylose maize starch, is robustly fermented by several different rodent models including young, male Sprague Dawley rats when fed a moderate or low fat diet. [20, 21] Although there has been phenotypic variation in body weight changes and fat accretion, male Sprague-Dawley, obese ZDF, and CD obese-prone rat models have distinctly demonstrated robust fermentation of Hi-Maize® 260 high amylose starch as indicated by enlarged ceca, decreased cecal pH, and increased production of SCFAs. [20–23] Martinez et al demonstrated that RS2 and RS4 can have different effects on the human colonic bacterial composition, which largely depended on the bacteria's accessibility to the different chemical structures of the RS types. [4] An important step in assessing the beneficial implementation of RS4 products is to perform a preclinical rodent study for determining the degree of fermentability. We hypothesized that the rats would respond to four novel RS4 products (Ingredion Incorporated) similarly as they had to RS2, high-amylose maize starch products. Therefore, the possible mechanisms of production of four novel RS4 products for total cecal fermentation in an *in vivo* rodent model were evaluated.

2 Materials and Methods

2.1 Animal Models

The study protocol for this work was approved by the LSU A&M IACUC committee as protocol #17–107. On arrival at the LSU Life Sciences Animal Care Facility, 3 wk old male, Sprague-Dawley rats (n=40) from Envigo (Houston, TX) were placed in a 1 week quarantine and fed a standard chow diet. After quarantine, rats were moved into stainless steel, hanging, wire-mesh cages in a climate-controlled room (21–22°C, 55% humidity) with a 12:12 light-dark cycle. Each cage contained a 3" x 6" hard plastic rest stop and PVC tube for "play". Rats were randomly assigned to 5 treatment groups (n=8), with similar mean body weights among groups.

2.2 The Diets

The four "unknown" RS4 starches were food grade modified starches provided by Ingredion Incorporated (Bridgewater, NJ). All other diet ingredients were purchased from Dyets Inc. (Bethlehem, PA). Five semi-purified diets were prepared with substitutions to the AIN-93G diet[24] using AMIOCA™ powder starch as the source of starch and the 4 unknown starches added with a reduction to the AMIOCA™ (Table 1). The starch products were not 100% resistant starch, and though the metabolizable energies of the four RS products were not known, it was assumed that the control diet (CON) had a greater energy content. We were informed post-study that RSA was VERSAFIBE™1490 modified potato starch, RSB was

VERSAFIBETM2470 modified corn starch, RSC was VERSAFIBETM2480 modified corn starch, and RSD was NOVELOSETM3490 modified tapioca starch (Table 2). The exact RS content of the starches could not be assessed because the Megazyme commercial kit (Chicago, IL) that is used to measure RS is not a reliable measure of RS4 due to underestimation. [25, 26] Our best estimate for RS was the fiber content of these diets, which is 85%, 65%, 80%, and 85%, respectively, reported as minimum total dietary fiber (TDF) on a dry-matter basis (AOAC 991.43, AOAC 2009.01, and AOAC 985.29, total dietary fiber methods). VERSAFIBETM1490 (RSA), VERSAFIBETM2480 (RSC), and NOVELOSETM3490 (RSD) are all distarch phosphate starches consisting of diester phosphate crosslinks between starch molecules [7, 8], and the VERSAFIBETM 2470 (RSB) is rendered indigestible by acid hydrolysis and heat treatment (Table 2). [7, 9] Rats were fed their respective diets for 3 weeks in their assigned treatments groups: control diet (CON) using AMIOCA® starch, VERSAFIBETM1490 (RSA), VERSAFIBETM2470 (RSB), VERSAFIBETM2480 (RSC), and NOVELOSETM3490 (RSD). Food intake (including an estimation of spillage) and body weights were measured twice weekly. Food cups were filled on the same two days and monitored throughout the week.

2.3 Dosage Information

The four novel RS4 starches were included at 10% of the weight of the diets. This is an estimate of 100g RS/1000kg of diet, but probably an overestimation because of fiber levels ranging from 65–85% of the diet. Although in past studies we have typically included 20% RS in diets to determine fermentation, we decided to assess these food-grade RS4 products as amounts that were approximately equivalent to the human fiber recommendation and that were more realistically achieved and tolerated in the human diet. [2, 27] Rats were given approximately 150g of food per week and allowed to eat their respective diets *ad libitum*.

2.3 Tissue Collection

Rats were humanely euthanized by isoflurane induction and cardiac exsanguination after 3 weeks on the diets. Blood was collected in tubes containing DPP-4 inhibitor for measurement of active GLP-1 using ALPCO ELISA kit (Salem, NH) as previously reported. [21, 22] The stomach, small intestine, cecum, and large intestine were separated and weighed individually full and empty. The full weights were combined and subtracted from the body weight at euthanasia to determine a disemboweled weight. The empty weights of the GI tract were added to the disemboweled weight to determine the emboweled body weight (EBW), and the latter was used to normalize total abdominal body fat (epididymal, perirenal, retroperitoneal fat pads). Total cecal contents were collected and frozen at –20°C for later pH and short-chain fatty acid (SCFA) measurements. After thawing, pH was measured with a solid phase pH electrode (Mettler Toledo SevenEasy™ pH Metter model S20) inserted into the contents, and total SCFA were extracted from ~0.5 g of cecal contents by addition of ~5 ml of distilled water using vigorous vortexing. Then ~1 ml of metaphosphoric acid containing the internal standard ethyl butyric acid (2 g/L) was added and this was followed by vigorous vortexing. The supernatant liquid collected after centrifugation was filtered into gas chromatography vials. The samples were run in duplicate with gas chromatography using an Alltech (Nicholasville, KY) Econo-cap EC-1000 with dimensions of 15 m x 0.53 mm, and a 100% polyethylene glycol acid film thickness of 1.20 micrometers.

2.4 Statistical Analysis

Data were analyzed as a one-way ANOVA of the independent variable “diet”. A *post hoc* Tukey analysis was then performed to determine significant differences among all comparison groups. A $p < 0.05$ was used for the significance level for the ANOVA and *post hoc* comparisons. Departures for the assumptions of normality of residuals of data and homogeneity of variance were tested. If variance of data was homogeneous, normality was tested with pooled variance, and if not, normality was tested with un-pooled variance. To account for possible type 1 error with multiple dependent variables analyzed, a Benjamini-Hochberg false discovery rate (FDR) test was used.^[28] This FDR test ranks the raw p values for the F value of the one-way ANOVAs and these were compared to a critical value (CV) which is determined by the formula: $(I/M)*Q$ with I = the rank for the p values from lowest to highest, M = number of dependent variables, and $Q = 0.05$. The highest p value that is less than CV and all ranked above are considered not false discovery.

3 Results

3.1 Statistical Results

Total cecal contents acetate, abdominal fat percent, and total cecal contents resistant starch data values were converted to log 10. For total cecum acetate and total cecal contents resistant starch, +1 was added to all values before conversion to log 10 to avoid negative log values. For all statistical analyses one rat, identified as RSD5, was not included because it lost 20 g body weight in 3 days prior to euthanasia. RSD5 had a biological reason for possible removal, which may be an apparent malfunction of the cage water bottle.

3.2 Markers of fermentation in cecum

Several measurements (Table 3) that are indicative of cecal fermentation had significant ANOVA F values. Rats that received novel starch diets RSB, RSC, and RSD had greater empty cecum weights (ECW, $p < 0.0001$), lower cecal contents pH ($p < 0.0001$), higher cecal contents wet weight ($p < 0.0001$), almost 2-fold greater total cecal contents acetate ($p < 0.0001$), and higher total cecal contents propionate ($p < 0.0051$) than the CON and RSA fed rats. The CON and RSA rats had similar results for all the above measurements except RSA rats had somewhat greater cecal contents wet weight than CON, but still significantly lower than rats fed RSB, RSC, and RSD. The ceca of RSB, RSC, and RSD fed rats were appreciably larger in size at necropsy (Supplemental Figure 1) when compared to the ceca of CON and RSA fed rats.

Two other indicators of fermentation, total cecal contents butyrate ($p < 0.1197$) and active GLP-1 ($p < 0.1743$), did not have significant ANOVA F values (Table 3). Although not statistically significant, the values for butyrate were numerically higher for RSA, RSB, and RSC compared to CON and RSD; and GLP-1 was numerically higher for RSB, RSC, and RSD compared to CON and RSA. A *post-hoc* power analysis for butyrate indicated that $n = 8$ rats had a power of 58.5% with the five means and standard deviation 0.03137. An $n = 12$ would have a power of 80.6%. The same type of analysis for GLP-1 indicated that $n = 8$ rats had a power of 55.2% with the five means and standard deviation 0.50124. An $n = 14$ would have a power of 84.8%.

3.2 Other phenotypic parameters

Four other dependent variables were measured to assess the effects of including resistant starches in diets (Table 4). These included resistant starch in cecal contents ($p<0.0001$), total food intake over three weeks ($p<0.4556$), EBW ($p<0.0393$), and total abdominal fat percent (abfat%, $p<0.0015$). RSB and RSC had significantly greater RS in cecal contents when compared to RSA and RSD. Total food intake was similar across all diet groups; however, RSA was the only group that had a significantly lower EBW than the CON group. All four RS groups had significantly lower abfat% when compared to the CON group.

3.3 Determination of false detection rate (FDR) for multiple dependent variables

To protect against a possible type 1 error in our statistical analyses, we used a Tukey *post hoc* analysis for multiple comparisons at the independent variable level. Use of multiple dependent variables also can increase the risk of type 1 errors, and therefore we used the Benjamini Hochberg, a FDR test, as a protective statistical tool (Table 5). [28]

4 Discussion

In the current study, the possible mechanisms of four novel RS4 products for total fermentation in an *in vivo* rodent model were evaluated. We expected Sprague-Dawley rats would ferment the RS4 within the cecum, but the novel products had three possible differences in their production that led to variable results. The first was the plant starch source of the product: potato, corn, or tapioca. The amount of RS4 was the second factor and this amount ranged from 65 to 85% of the product. Because an assay for measurement of RS4 is lacking, previous publications have reported total fiber as a surrogate marker for RS4. [25–26] If total fiber is an accurate surrogate for RS4, then none of the four RS4 products comprised 100% of the 10 g per 100 g of diet. At the start of the study, these products were unknowns and we used each one at 10% of the weight of the diet. The 10% amount is approximately equivalent to the fiber requirement for humans. [27] Thus, the amounts of RS4 as total fiber were well within the human fiber requirement in our rat model. The third difference in production of the RS4 products was the modification of the starch to produce RS4, which was by phosphate cross-linking or acid hydrolysis and heat treatment. [7–9] We therefore propose these three mechanistic differences in the RS4 products that might determine fermentability, which include: the origin of the starch, the amount of starch available for fermentation, and the different methods of production used for the RS4 products.

Before our assessment of fermentation in rats, clinical trials in humans demonstrated that a tapioca-based RS4 starch (Cargill, Inc.) as well as the VERSAFIBE™ 1490 modified potato starch (RSA) and VERSAFIBE™ 2470 (RSB) modified corn starch, when included in bakery products such as a breakfast bar, cookie, and muffin top, respectively, reduced postprandial glucose and insulin responses in healthy adults. [29, 8, 9] However, the degree of fermentability, and thus the mechanistic actions on colonic health and the microbiota, remained to be fully elucidated. The recent *in vitro* study by Erickson et al demonstrated that RS4 products consisting of modified tapioca, potato, and corn starches were fermentable and produced SCFA comparable to a slowly and rapidly fermented carbohydrate control i.e.

polydextrose and FOS, respectively. [7] A porcine *in vitro* fermentation model revealed that different starch substrates, including native and recrystallized potato and maize starches, gave rise to significantly different microbial communities.[30] Moreover, preliminary work in humans by Deehan et al suggests that RS4 supplementation effects on microbiome modulation is dependent on the chemical structure (and origin) of the starch product. RS4 products with corn and tapioca starches enriched different OTUs of bacterial taxa and led to an increase in butyrate and propionate, respectively, while the potato derived RS4 did not significantly alter the microbiome. [31] While we did not perform microbial sequencing for this study, we did see similar results in that the RS4 products, comprised of similar modified corn and tapioca starches, did readily ferment in the cecum of rats. Lack of significant markers of fermentation of the RSA produced with the modified potato starch might align with Deehan et al, but microbial sequencing would need to be performed. [31] In fact, *in vitro* studies have shown that products made with 1,3 glycosidic bonds between glucose molecules were not digestible, but the glucans were digested during *in vivo* studies in chickens, rats, and humans (unpublished data). This further emphasizes the importance of not just performing *in vitro* analyses, but always performing *in vivo* studies to assess true fermentation and physiological effects of a particular product. [7]

Our aim was to use an amount of RS that was estimated to be equivalent to the recommendation for human consumption, approximately 10% weight of diet maximum [2, 27]. The amount of RS4 in the diets was lower than 10% considering that the total fiber levels used as surrogate values for RS content were 65–85% of the RS4 products. We found in this study that the profile of major SCFA in cecal contents was significantly different for acetate and propionate, but not for butyrate. It was anticipated that total cecal contents butyrate would also be significantly increased for the resistant starch groups based on previous studies with resistant starch type 2 high-amylose maize at 20% or higher of the diet. [2, 18, 20] Our lack of significance among all groups for butyrate appears to be a lack of statistical power and, more notably perhaps, a lower dose of RS. As this was a pilot study, an n = 8 was used that possibly would have demonstrated significant differences for RS groups versus control. However, a *post hoc* power analysis revealed that there was low power with n=8 rats for cecal butyrate analysis, and that an n = 12 was needed to reach an acceptable 80% power. Furthermore, our previous studies that observed increased total cecal butyrate amounts used mechanistic, proof-of-concept amounts of RS2 at ~20% of the weight of the diet, whereas we likely had less than 10% RS4 in these diets. [2] We also did not observe significant differences among groups for serum active GLP-1; however, RSB, RSC, and RSD groups had greater numerical values than RSA and CON. In light of a low statistical power, the lower amount of RS in the diet (<10%) may have affected fermentation of RSA as well as precluded the demonstration of significant differences in butyrate and GLP-1 levels across all diet groups.

Finally, we speculated whether the methods of production of the RS4 starches affected the degree of fermentation. RSA, which is Versafibe 1490™, contained approximately 85% RS based on the total fiber content and did not appear to ferment [32]; however, RSB, which is Versafibe 2470™, contained approximately 65% RS and was robustly fermented. This suggests that bacteria utilization of the starches is not completely dependent on amount of RS4 in the diet alone, but also on the structural differences of the starches. RSA, RSC, and

RSD were chemically modified by distarch phosphate crosslinks, while RSB underwent acid hydrolysis and heat treatment. [7–9] Without application of chemical constituents or cross-linkages, [9] acid hydrolysis and heat treatment likely produces RS4 by breaking existing chemical bonds by hydrolysis followed by alteration of the bonds with heat which cause a structural change that renders the starch indigestible. RS4 produced with chemical constituents partially blocks digestive enzyme accessibility, but has regions within the starch that are accessible to bacterial hydrolysis and fermentation. [10] In contrast, RS4 that is highly cross-linked does not swell during cooking and remains in a granular form that is resistant to digestion and reported by Birt et al to not undergo bacterial fermentation. [10] However, in our study, RSC and RSD considered cross-linked by distarch phosphates in a similar manner to RSA, appeared to be readily fermented by bacteria in the rat cecum. We therefore propose that the cross-linked structure of the potato starch, as opposed to the corn and tapioca starches, appear to hinder bacterial accessibility to a greater degree.

Furthermore, these structural differences may affect speed of fermentation and give rise to distinct bacterial communities at different rates throughout the GI tract. Martinez et al. suggests that gut bacteria may differ in their ability to adhere to the starch granules of RS2 and RS4, which could be an important and necessary step in the preliminary response of the bacteria to these starches. [4] Feeding RS2 vs. RS4 to humans revealed shifts in fecal microbial communities and the rate in which certain bacterial species increased over time suggesting significant temporal dynamics between starch substrates. [4] As referred to earlier, utilization of a porcine *in vitro* fermentation model to assess several different starch substrates demonstrated that different RS structural properties led to distinct microbial communities with different fermentation temporal patterns, enzyme kinetics, and SCFA profiles. [30] We questioned if RSA was slowly fermented throughout the GI tract rather than predominantly in the cecum. Aoe and colleagues report that RS in BARLEYmax, a barley line with 3 fermentable fibers, has the slowest rate of fermentation in rats when compared to fructans and β -glucans. SCFA concentrations following fermentation of BARLEYmax were found to be significantly higher in the distal colon of male Sprague-Dawley rats compared to the proximal colon. Possibly, distal colonic RS fermentation of BARLEYmax in rats is slowed by the other components in the product, but the concentration of SCFA was still 2-fold greater in the cecal digesta than in the distal colonic digesta. [33]. Moreover, our previous studies have observed major fermentation of resistant starch type 2 in the cecum of rats. [17–20] In the current study, the empty distal large intestine (large intestine without the cecum) weights for the CON and RSA groups were similar, but significantly greater than for RSB, RSC, and RSD (Supplemental Table 1). Thus, it appears as if RS2 in previous studies and RS4 in the current study are primarily fermented in the cecum of the rat, and it is likely that assessing SCFA amounts in the colonic contents would not have provided different results for RSA.

It is important to note that we cannot conclude that RSA, a modified potato starch, which appeared to be non-fermentable, was digested. The Amioca™ powder starch in the control group was digestible, and it was anticipated that the rats would have higher abfat% due to higher energy extraction. Yet, the RSA group had the lowest EBW of all groups and lower abfat% compared to CON. If RSA had been digested, we would have expected those biometric markers to be more similar to CON (Table 4). Testing carbohydrates remaining in

the cecal contents or feces could have been performed to clarify digestibility, but our group was focused on fermentation responses.

Concluding Remarks

This study has shown that RS4 products that are produced from different starch origins with varying amounts of RS4 content and different methods of production are not uniformly fermented in an *in vivo* model. These production differences among the RS4 starch products may not offer the same potential health benefits for universal recommendations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

| | |
|---------------------------|---|
| ABF% | abdominal fat percent |
| AIN-93G | American Institute of Nutrition 1993 growth rodent diet |
| EBW | emboweled body weight |
| ECW | empty cecum weight |
| GLP-1 | glucagon-like peptide 1 |
| RS | resistant starch |
| RS4 | resistant starch type 4 |
| RSA, RSB, RSC, RSD | Resistant Starch Type 4 novel starches A, B, C, and D |
| SCFA | short-chain fatty acids |

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Table 1.Diets for the study ^{a)}

| Ingredients (g) | | Ingredient Description | CON | RSA | RSB | RSC | RSD |
|-----------------|---------------------|--------------------------------|-------|-------|-------|-------|-------|
| Macro Mix | Starch | AMIOCA™ | 530.6 | 430.6 | 430.6 | 430.6 | 430.6 |
| | | Resistant Starch ^{b)} | 0.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| | Sugar | Sucrose | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| | Protein | Casein | 200.0 | 200.0 | 200.0 | 200.0 | 200.0 |
| | Fiber | Cellulose | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 |
| Fat | Soybean Oil | | 70.0 | 70.0 | 70.0 | 70.0 | 70.0 |
| Micro Mix | Mineral Mix AIN-93G | | 35.0 | 35.0 | 35.0 | 35.0 | 35.0 |
| | Vitamin Mix | | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| | Choline Chloride | | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 |
| | L-Cystine | | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| Total | | | 1000 | 1000 | 1000 | 1000 | 1000 |

^{a)}Diets based off AIN-93G purified diets for laboratory rodents by Reeves et al [25].

^{b)}RS4 products: RSA was VERSAFIBE™1490 modified potato starch, RSB was VERSAFIBE™2470 corn starch, RSC was VERSAFIBE™2480 modified corn starch, and RSD was NOVELOSE™3490 modified tapioca. These RS4 products were supplied by Ingredion Incorporated.

Table 2:Resistant starch products ^{a)}

| Test Samples | Lot number | Ingredients | TDF % ^{b)} | Production Method ^{c)} |
|--------------------------|-----------------|--|---------------------|---|
| Control | Batch LG5440 | Amioca™ powder starch | 0% (Theoretically) | Isolated from waxy corn that has 100% amylopectin ^{d)} |
| Resistant Starch A (RSA) | Lot: 17J-922 | VERSAFIBE™ 1490 modified potato starch | 85% | Distarch phosphate with diester phosphate crosslinks |
| Resistant Starch B (RSB) | Lot: LFI0022 | VERSAFIBE™ 2470 modified corn starch | 65% | Acid hydrolyzed and heat treatment |
| Resistant Starch C (RSC) | Lot: 0001228644 | VERSAFIBE™ 2480 modified corn starch | 80% | Distarch Phosphate |
| Resistant Starch D (RSD) | Lot: FGB2008 | NOVELOSE™ 3490 modified tapioca starch | 85% | Distarch Phosphate |

^{a)} Starches were provided by Ingredion Incorporated and RS amount and metabolizable energies for each product were unknown to the authors prior to the end of the study. Each starch was included as 10% of the weight of the diet.

^{b)} Total digestible fiber percentage was used as a surrogate for estimating RS4 content of the starches. TDF% was taken from product technical specification sheets supplied by Ingredion Incorporated [32] or through direct correspondence with Ingredion Incorporated.

^{c)} Production methods, or chemical modifications, to produce RS4 were based on similar Ingredion Incorporated products used by Erickson et al and Stewart et al [24–26]. However, the TDF from those studies could not be used for our study since lot numbers could have been different.

^{d)} Data referenced from M.J. Keenan, J. Zhou, M. Hegsted, C. Pelkman, H.A. Durham, D.B. Coulon, R.J. Martin, *Adv. Nutr.* **2015**, *6*, 198.

Table 3.Dependent variables that indicate fermentation effects^{a),b)}

| Groups | CON | RSA | RSB | RSC | RSD |
|--|------------------------|--------------------------|------------------------|--------------------------|----------------------------|
| Empty cecum weight (g) | 0.49±0.01 ¹ | 0.53±0.04 ¹ | 0.81±0.05 ² | 0.72±0.03 ² | 0.78±0.05 ² |
| Cecal contents pH | 8.21±0.18 ¹ | 8.08±0.18 ¹ | 6.34±0.18 ² | 6.49±0.18 ² | 5.95±0.19 ² |
| Cecal contents wet weight (g) | 2.58±0.15 ¹ | 3.85±0.29 ² | 9.09±0.60 ³ | 7.50±0.39 ³ | 8.50±0.68 ³ |
| Total cecal contents acetate (mmol/cecum) ^{c)} | 0.16±0.01 ¹ | 0.20±0.02 ¹ | 0.53±0.06 ² | 0.35±0.02 ² | 0.48±0.05 ² |
| Total cecal contents propionate (mmol/cecum) ^{c)} | 0.04±0.00 ¹ | 0.05±0.00 ^{1,2} | 0.11±0.02 ³ | 0.09±0.01 ^{2,3} | 0.05±0.01 ^{1,2,3} |
| Total cecal contents butyrate (mmol/cecum) ^{c)d)} | 0.04±0.00 | 0.06±0.01 | 0.06±0.02 | 0.07±0.02 | 0.03±0.01 |
| GLP-1 (pM) ^{d) e)} | 1.32±0.19 | 1.40±0.19 | 1.60±0.19 | 1.61±0.19 | 1.98±0.20 |

^{a)} The groups are CON = control group, RS = resistant starch, and A through D are four unknown resistant starches prior to the end of the study; data reported as means±SEM; values with different superscript numbers are significantly different at $p < 0.05$.

^{b)} Different superscript numbers across rows indicate statistical significance determined using a Tukey *post-hoc* analysis at $\alpha = 0.05$.

^{c)} mmol/cecum = millimoles in the entire cecal contents and amounts of short-chain fatty acids per cecum are identical when using either wet or dry weights of cecal contents.

^{d)} Values for non-significant fermentation indicators

^{e.)} pM = picomolar and active GLP-1 was measured.

Table 4.Values for other biometric data ^{a), b)}

| Groups | CON | RSA | RSB | RSC | RSD |
|--|--------------------------|--------------------------|----------------------------|----------------------------|----------------------------|
| Resistant starch (cecal contents, g/100 g) ^{c)} | 0.08±0.04 ¹ | 0.30±0.02 ² | 1.00±0.16 ³ | 1.38±0.21 ³ | 0.22±0.03 ^{1,2} |
| Total food intake (3 weeks, g) | 379.05±5.05 ¹ | 376.88±5.05 ¹ | 367.54±5.05 ¹ | 369.73±5.05 ¹ | 371.05±5.40 ¹ |
| EBW (g) ^{d)} | 266.36±5.13 ¹ | 243.95±5.13 ² | 248.96±5.13 ^{1,2} | 249.67±5.13 ^{1,2} | 256.47±5.48 ^{1,2} |
| Abfat% ^{e)} | 1.84±0.07 ¹ | 1.42±0.03 ² | 1.51±0.09 ¹ | 1.49±0.10 ² | 1.41±0.07 ² |

^{a)} The groups are CON = control group, RS = resistant starch, and A through D are four unknown resistant starches. Values with different superscript numbers are significantly different at $p < 0.05$.

^{b)} Different superscript numbers across rows indicate statistical significance determined using a Tukey *post-hoc* analysis at $\alpha = 0.05$.

^{c)} Resistant starch was measured using the Megazyme Company assay (Chicago, IL). However, the assay under reports resistant starch values for RS4.

^{d)} EBW = emboweled body weight, which is the body weight minus the full GI tract weight plus the empty GI tract weight.

^{e)} Abfat% = abdominal cavity fat (epididymal, perirenal, retroperitoneal) normalized with division by EBW.

Table 5.Benjamini-Hochberg false discovery rate test ^{a)}

| Dependent variables | ANOVA F p values ¹ | I | M | Q ¹ | I/M*Q=CV |
|--|-------------------------------|----------|----|----------------|----------------------------|
| Empty Cecum weight (g) | 0.0001 | 1 | 11 | 0.05 | 0.0045 |
| Cecal Contents pH | 0.0001 | 2 | 11 | 0.05 | 0.0091 |
| Cecal Contents wet weight (g) | 0.0001 | 3 | 11 | 0.05 | 0.0136 |
| Resistant Starch (g/100 g) | 0.0001 | 4 | 11 | 0.05 | 0.0182 |
| Cecal Contents Total Acetate (mmol/cecum) | 0.0001 | 5 | 11 | 0.05 | 0.0227 |
| Abdominal fat % (Abfat%: Abfat/EBW*100) | 0.0015 | 6 | 11 | 0.05 | 0.0273 |
| Cecal Contents Total Propionate (mmol/cecum) | 0.0051 | 7 | 11 | 0.05 | 0.0318 ^{a)} |
| Emboweled Body Weight (EBW, g) | 0.0393 | 8 | 11 | 0.05 | 0.0364^{b)} |
| Cecal Contents Total Butyrate (mmol/cecum) | 0.1197 | 9 | 11 | 0.05 | 0.0409 |
| Serum Glucagon-like Peptide-1 (GLP-1, pM) | 0.1743 | 10 | 11 | 0.05 | 0.0455 |
| Food Intake (g) | 0.4556 | 11 | 11 | 0.05 | 0.05 |

^{a)} Benjamini-Hochberg test was described in McDonald et al [30], alpha for the one-way ANOVA for the study was $p < 0.05$, Q was chosen as 0.05 as recommended by our statistician. Highest p value that is less than CV and all ranked above are considered not false discovery.

^{b)} Significantly different, but borderline false discovery.