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Simultaneous delivery of antibiotics neomycin and ampicillin in drinking water inhibits fermentation of resistant starch in rats

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Abstract

Scope—Antibiotics ampicillin 1g/L and neomycin 0.5g/L were added to drinking water before or during feeding of resistant starch (RS) to rats to inhibit fermentation.

Methods and Results—In a preliminary study antibiotics and no RS were given prior to rats receiving a transplant of cecal contents via gavage from donor rats fed RS (without antibiotics) or a water gavage before feeding resistant starch to both groups. Antibiotics given prior to feeding RS did not prevent later fermentation of RS regardless of either type of gavage. In the second study antibiotics were given simultaneously with feeding of RS. This resulted in inhibition of fermentation of RS with cecal contents pH >8 and low amounts of acetate and butyrate. Rats treated with antibiotics had reduced *Bifidobacteria spp.*, but similar *Bacteroides spp.* to control groups to reduce acetate and butyrate and preserve the production of propionate. Despite reduced fermentation, rats given antibiotics had increased glucagon-like peptide 1 (GLP-1) and cecum size, measures that are usually associated with fermentation.

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Conclusions—A simultaneous delivery of antibiotics inhibited fermentation of RS. However, increased GLP-1 and cecum size would be confounding effects in assessing the mechanism for beneficial effects of dietary RS by knocking out fermentation.

Keywords

Antibiotics; resistant starch; fermentation; glucagon-like peptide 1; short-chain fatty acids

1. Introduction

In previous reports of beneficial effects of dietary RS, reviewers would not allow a claim of cause and effect, but only to state an association. In this current scientific age with use of knockout, knockdown, or knock in, intervention studies without these approaches do not provide acceptable cause and effect. Therefore, the objective of this research was to develop a method for use in hypothesis-testing to determine if health benefits of resistant starch (RS) reported [1-4] were the result of fermentation or if its presence in the gut without fermentation has an effect. Additionally, we hypothesized that an initial knockdown may persist over the long-term [5] and produce a non-responder model that could be used for investigating treatments for individuals that are reported not to ferment RS well [6].

Two specific uses of antibiotics in rodents are to successfully improve metabolic endotoxemia associated with obesity [7] or to reduce microbiota for creation of a niche for successful transplant of cecal contents [8]. Cani et al used neomycin and ampicillin [7], and Manichanh et al. [8] used vancomycin and imipenem. Both of these studies delivered antibiotics by including them in the drinking water. In the current study we are reporting the use of ampicillin and neomycin to determine if fermentation was inhibited. These two antibiotics were chosen because they are each large spectrum with ampicillin targeting mainly gram-positive bacteria [9] and neomycin targeting mainly gram-negative bacteria [10]. Their use is convenient because the doses were reported as g/L [7] as opposed to Manichanh et al [8] that give the dose of two antibiotics each as 50 mg/kg body weight per day and relies on estimating water consumption for the rats.

Fermentation of RS involves attachment of *Bacteroides thetaiotaomicron* (Gram negative, [11]), *Bifidobacterium longum* (Gram positive, [12]), and some *Lactobacillus spp* (Gram positive, [13]) to the surface of starch molecules. *Bacteroides spp* fermentation products are acetate, propionate and succinate; while products of *Bifidobacterium spp* and *Lactobacillus spp* are lactate and acetate [14]. Lactate and acetate are used by bacteria in *Clostridium cluster IV* and *Clostridium cluster XIV* (Gram positive, [15]) to produce butyrate [16, 17].

Two studies were conducted to determine if ampicillin (1g/L) and neomycin (0.5g/L), large-spectrum antibiotics, in drinking water were able to prevent fermentation of RS by using them either prior to or during feeding of RS diet. In Study 1, preliminary study, we measured fermentation markers, pH of cecal contents and empty cecum weights (ECW), to determine if use of antibiotics prior to feeding RS would subsequently prevent fermentation of RS. This was to determine possible long-term effects [5] to produce a non-responder model. However, rats were able to robustly ferment RS when antibiotic treatment was given and then stopped prior to feeding RS. Since we did not produce a non-responder model, we

ended study 1 and proceeded to Study 2, where we determined if antibiotics would be able to reduce fermentation when RS was fed at same time as antibiotic treatment. In the second successful study, other measurements were added.

In the second study we also added a group fed RS in a high fat diet because Cani et al. had previously used ampicillin and neomycin to decrease inflammatory endotoxemia associated with a high fat diet [7]. We hypothesized the antibiotics may improve fermentation of RS by reducing bacterial types that increase with feeding of a high fat diet. Previous research done in our lab demonstrated high fat in the diet of rats partially attenuated the ability to ferment RS [18]. However, antibiotics prevented fermentation of RS in either low or high fat diets, when fed simultaneously with antibiotics.

Materials and Methods

2.1 Animals and diets

Both studies were approved by IACUC at Louisiana State University as protocol 13-049. Male Sprague Dawley rats were purchased from Harlan Laboratories Inc. (Indianapolis, IN) at 6 weeks old, and maintained in quarantine for 1 week on a chow diet. After quarantine, rats were stratified according to their body weight. Rats were housed individually in wire bottom cages at 21-22°C, 55% humidity, and 12:12 hour light-dark cycle. Rats had free access to food and water. Body weight, food intake, and food spillage were measured twice a week.

Study 1—Twenty-nine rats were used in this study in two phases. In phase 1 there were two groups: high resistant starch (HI-MAIZE® resistant starch, RS, 27.8% weight) in diet and no antibiotics (RS+NAB, n=10) and low RS (NRS+AB, n=19) AIN-93M diet [19], with antibiotics in drinking water (ampicillin 1g/L, neomycin 0.5g/L). Ampicillin (A0166) and Neomycin (N6910) used were tested in cell culture by Sigma (St. Louis, MO). The rats were fed diets (Supplemental Table 5) for 4 weeks.

At end of first phase, the two groups were each subdivided into two subgroups. Five rats of the nineteen from NRS+AB and five rats from RS+NAB rats were euthanized to measure empty cecum weight and cecal contents pH. The remaining five in RS+NAB were euthanized for pooling of cecal contents for gavage in phase 2 to seven rats from NRS+AB. These n=7 rats were designated as high RS and cecal contents gavage (RS+CG). Another seven rats from NRS+AB received water gavage and designated as RS+WtG. The gavage followed by feeding high RS (Supplemental Table 5) and constituted phase 2. These fourteen rats were presumed to have knockdown of their microbiota, and hypothesized they would not ferment RS unless they received the cecal contents gavage instead of the water gavage. The volume for the gavages was 5 ml of 1:10 diluted cecal contents in saline solution. Phase 2 lasted 3.5 weeks.

Study 2—Twenty-four rats were fed diets for four weeks. Diets (Table 1) were (1) low fat with high RS (LFRS), (2) high fat (42% of energy) with high RS (HFRS), and (3) low fat with amylopectin control corn starch with no resistant starch (LFNRS). High RS was 23.5% by weight of diet. Two groups received the same antibiotic treatment as in Study 1 with

designations AB=antibiotic and NAB=no antibiotic. The four groups (n=6) were LFRS+AB, HFRS+AB, LFNRS+NAB, and LFRS+NAB, where LFNRS+NAB was considered the negative control and LFRS+NAB was considered the positive control. One rat cecum was used for histology leaving n=5 for other cecal analyses. For *Bifidobacterium spp.* analysis the HFAB group had n=4.

2.2 Starches

The RS used in both studies was RS type 2 which is naturally found in raw green bananas and the natural corn hybrid of HI-MAIZE RS that is high in amylose and low in amylopectin as a result of low branching enzyme [20]. The presence of the starch granular structure limits access of the starch to amylase enzyme [20]. RS in the HI-MAIZE RS was analyzed by Ingredion Incorporated using the modified Englyst assay [21]. There are four other types of RS. These include RS1, 3, 4, and 5. RS1 is found in whole grains that are not overly processed and the whole grain structure limits amylase enzyme access [20]. Retrograded RS3 is found in cooked and then cooled potatoes as found in potato salad. This retrogradation creates a network with increased density of RS3 crystallites as opposed to granules that limits access of amylase enzyme [22]. RS4 is a modified starch created chemically by either adding substituent groups or linkage of starch molecules that limit access of the amylase enzyme to the starch [23]. Another RS is RS5 that is a combination of amylose and free fatty acids that forms a structure that also prevents access to amylase enzyme [23].

The control starch used in diets mixed by our lab in studies is AMIOCA® cornstarch and is called waxy starch. This starch product is 100% amylopectin and 100% digestible and is recommended as the starch for control diets (Ingredion Incorporated; Bridgewater, NJ).

2.3 Procedures

In studies 1 and 2, rats were euthanized by cardiac puncture exsanguination followed by removal of the heart under inhalation of isoflurane anesthesia. The gastrointestinal tract (GI) was removed from esophagus to anus, and then it was divided into stomach, small intestine, cecum and large intestine, and weighed full and empty. Fat pads (peritoneal, retroperitoneal, and epididymal) were weighed. Abdominal fat percentage (ABF%) was calculated by dividing abdominal fat by body weight of rats with GI tract contents removed. Cecal contents were frozen in liquid nitrogen for measurement of pH, and in Study 2 short chain fatty acids (SCFAs), and targeted bacterial genera that ferment resistant starch. For Study 2, blood samples were collected by cardiac puncture into tubes with DPP4 inhibitor for measurement of GLP-1 active with ELISA kit (ALPCO, NH) and one full cecum per group was used for histology of cecum wall.

2.3.1 Cecal contents pH and SCFAs analysis—For studies 1 and 2, cecal contents were thawed and ~0.5 g of each rat's wet sample were homogenized in 5 ml of distilled water, for pH measurements. For study 2, each sample was acidified with 1 ml of a 25% (w/w) solution of metaphosphoric acid containing 2 g/L 2-ethyl-butyric acid as internal standard for SCFAs. Solids were separated by centrifugation and filtration, and supernatant

transferred to GC auto-sampler vial. SCFAs were quantitatively determined by gas chromatography described previously [18].

2.3.2 Bacterial DNA extraction

Study 2: Approximately 200 mg of each rat's cecal contents were placed in 2 ml screw-cap tube with ~ 300 mg of 0.1 mm Zirconia Silica beads (BioSpec Products Inc, OK), then 100 µl of Lysis buffer prepared with 5 M NaCl, 1 M Tris-HCl (pH 8), 0.5 M EDTA (pH 8), distilled autoclaved water and Lysozyme (Thermo Scientific, IL) were added. The tubes were vortexed, and incubated for 30 minutes at 37°C. Then 1 ml of InhibitEX buffer from QIAamp® Fast DNA Stool Mini kit (QIAGEN, CA) was added. The mixture was then homogenized two times using a FastPrep®-24 Instrument (MP Biomedicals, OH) for 60 seconds at speed setting 6.5 m/s. The suspension was then heated at 95°C, shaken at 250 rpm for 5 minutes, then vortexed 15 seconds and centrifuged for 3 minutes at 14,000 rpm. Finally, DNA was extracted from supernatant by following QIAamp® Fast DNA Stool Mini kit protocol. Purified DNA was quantified using a NanoDrop® Spectrophotometer and diluted to 1 ng/µl for storage at -80°C.

2.3.3 Quantitative real-time PCR (qPCR)—In study 2, ABI PRISM 7900 Sequence Detection System, and SDS 2.4 Software (Life Technologies, NY) were used to perform SYBR® Green method of qPCR. Published primers were used to measure several bacterial genera that included *Bacteroides* spp. [24], *Lactobacillus* spp. [25], *Bifidobacterium* spp. [26], *Clostridium* cluster XIV a, b [27], and universal primers [28] were used for total bacteria. Primer sequences are listed in Supplemental Table 1. All reactions were performed using sterile MicroAmp® Optical 384-well Reaction Plates with Barcode and sealed with MicroAmp® Optical Adhesive Films (Life Technologies, CA). Reactions were performed in triplicates with 5µl of 2X SYBR Green Master Mix (Life Technologies, CA), 0.5 µl of each primer at 10 µM (Integrated DNA Technologies, IA), 0.5 µl of 250 mg/ml bovine serum albumin (BSA), 0.5 µl of nuclease free water and 3 µl of DNA template in 10 µl total volume.

The specificity of primer sequences were checked with GenBank by blast search of NCBI (<http://www.ncbi.nlm.nih.gov>) September 30, 2015. The cycling conditions for all were one cycle of 95°C 10 minutes, then 40 cycles of 95°C 15 seconds, primer-specific annealing temperature (Supplemental Table 1) for 1 minute, 78°C for 40 seconds, and after amplification a dissociation step was included (95°C for 15 sec, 60°C for 15 sec, 95°C for 15 sec).

Primers for *Lactobacillus* spp. [25] and *Clostridium* cluster XIVa, b [27] were initially designed for use with Taqman® probe, but with added specificity using the probe we were unable to detect amplification and used primers with SYBR green. *In silico* tests against GenBank for primers for *Lactobacillus* spp. resulted in 89% specificity and for *Clostridium* cluster XIVa, b there was 60% specificity excluding chloroplasts and clones. Primers for *Bacteroides* spp. and *Bifidobacterium* spp. had 57% and 75%, specificity, respectively. Thus, our qPCR results are believed to largely reflect the effects on genera of interest.

Several bacterial strains representing genera measured were cultured in specific broths (Supplemental Table 2). Serial dilutions of each type of cultured bacteria were made by several dilutions with PBS (Supplemental Table 2). The final dilutions of bacteria were spread on agar plates using 100 μ l. Colony Forming Units (CFU) were determined for serial dilutions and converted to log CFU/ml for the undiluted bacteria in broth. This value was used to determine log CFU/ml for dilutions for qPCR standard curves. These curves were produced using serial 1/4 dilutions of DNA extracts. DNA was extracted from 1 ml of undiluted broth and standard curves constructed as Ct versus log CFU/ml (Supplemental Table 3).

2.3.4 Resistant starch assay—For study 2, cecal contents (100 $\pm$ 5 mg) were analyzed for RS content. This was measured as g/100 g following the protocol of the RS Assay Kit (Megazyme Inc, Chicago, IL).

2.3.5 Histology of cecum wall—For study 2, one cecum per group was placed with contents into a jar with 100 ml of 10% formalin for 72 hours. Next, the bottom portion was cut and placed into histology cassettes. Cross-sectional slices of the cecum wall were visualized with Hematoxylin and Eosin stain. Pictures were made using a NanoZoomer-SQ Digital slide scanner (Hamamatsu, Japan) at 20X magnification. Images from tissue samples were analyzed using NanoZoomer Digital PathologyView2 Software. The heights of mucosal, submucosal, and muscularis layers were measured in three different locations per slide image and then averaged.

2.4 Statistical Analysis

Statistical Analysis Software SAS® version 9.3 MIXED procedure was used for one-way ANOVA followed by F-protected LSD. Equal variance, normal distribution, and outliers were identified by UNIVARIATE procedure. If normality assumption was not met, data were transformed to log10. A $p < 0.05$ was considered statistically significant. In Study 2, propionate data was transformed to log 10 for statistical analyses. Data were presented in their original form and expressed as means $\pm$ pooled standard error.

3. RESULTS

3.1 Study 1

3.1.1 Cecal contents pH and Empty Cecum Weight (ECW)—In phase 1 (Supplemental Table 6), the group fed RS (RS+NAB) showed greater fermentation than the group that received antibiotic treatment and no dietary RS (NRS+AB) with lower pH of cecal contents ($p < 0.0002$). The ECW for the RS+NAB rats were not significantly different ($p = 0.07$) from NRS+AB rats given antibiotics without RS.

In phase 2 (Supplemental Table 6), rats in the RS+NAB group from phase 1 were considered a positive control and rats in the NRS+AB the negative control for comparison with rats in the RS+WtG and RS+CG groups for pH, but not for ECW as the RS+NAB and NRS+AB rats were younger (11 weeks old) than rats in the RS+WtG and RS+CG groups (14.5 weeks old) at euthanasia. The positive control cecal contents pH was significantly lower than RS

+WtG ($p<0.002$) or RS+CG ($p<0.024$) groups. However, RS+WtG ($p<0.0001$) and RS+CG ($p<0.0001$) had a lower cecal contents pH than the NRS+AB rats not fed RS and treated with antibiotics. Additionally, ECW was not significantly different between RS+WtG and RS+CG.

3.2 Study 2

3.2.1 Cecal contents pH, ECW, SCFAs and RS in cecal contents, serum GLP-1, and ABF%—Results (Table 2) demonstrated that the LFNRS+NAB group (negative control) did not have a significantly different pH of cecal contents compared to groups fed resistant starch and concurrently treated with antibiotics (LFRS+AB, $p=0.35$ and HFRS+AB, $p=0.99$). The LFRS+NAB had the lowest cecal contents pH (all $p<0.0001$).

ECW of groups given antibiotic treatment, LFRS+AB ($p<0.0001$) and HFRS+AB ($p<0.0005$), were significantly greater than the negative control group (LFNRS+NAB); and ECW of LFRS+AB ($p=0.1940$) was not significantly different from the positive control group (LFRS+NAB) or from the HFRS+AB group ($p=0.16$). However, the ECW for the HFRS+AB group was significantly lower than the positive control group ($p<0.01$).

Acetate production for the LFRS+AB group was significantly lower than that of the positive control ($p<0.014$), significantly greater than the negative control ($p<0.016$), and not different from the HFRS+AB group ($p=0.96$, Table 2). Thus, there was some increased production of acetate with the combination of RS and antibiotics with both low and high fat diets, but the antibiotics reduced production compared to the positive control. Butyrate production for the two groups treated with antibiotics was lower ($p<0.02$) than the negative control group and the negative control group had lower ($p<0.0008$) butyrate production than the positive control group (Table 2).

Propionate production was similar for the two groups with low fat diets with RS regardless of antibiotic treatment ($p=0.44$). The high fat diet with antibiotic treatment and RS had less propionate production ($p<0.03$) than the low fat group with antibiotic treatment and RS, but had similar production ($p=0.89$) as the positive control group. All three groups with RS had greater propionate production than the negative control group ($p<0.001$).

Groups given antibiotic treatment, LFRS+AB ($p<0.0001$) and HFRS+AB ($p<0.0001$), had about double the amount of RS in cecal contents than the positive control group (LFRS+NAB, Table 2). This demonstrated that at the mechanistic dietary levels of RS used in this study and previous studies (1-3) about half of RS is not fermented. The LFRS+AB and HFRS+AB were not significantly different from one another ($p<0.6897$) in amount of RS in cecal contents.

Serum active GLP-1 (Table 2) for groups given antibiotic treatment, LFRS+AB ($p<0.011$) and HFRS+AB ($p<0.0006$), were greater than the negative control group (LFNRS+NAB), and the positive control (LFRS+NAB) had lower ($p<0.02$) active GLP-1 than the HFRS+AB and greater amounts ($p<0.0006$) than the negative control group. However, the positive control group was not significantly different from the LFRS+AB group ($p=0.08$) because the

LFRS+AB group had greater individual variation than the three other groups (SEM: LFRS+AB, 0.36; HFRS+AB, 0.19; LFNRS+NAB, 0.06; LFRS+NAB, 0.14,).

The ABF% (Table 2) was greatest for the group with a high fat diet. The HFRS+AB group had significantly greater ABF% than the LFRS+AB ($p<0.045$), negative control group ($p<0.03$) or positive control group ($p<0.02$).

3.2.2 Histology—The cecal walls (Supplemental Table 4 and Supplemental Figure 1) of the positive control (LFRS+NAB) and the two groups treated with antibiotics (HFRS+AB and LFRS+AB) were all numerically similar and all numerically greater than the cecal wall of the negative control (LFNRS+NAB).

3.2.3 Bacterial groups—The total amount of bacteria (Table 3, universal 16S primers) was numerically greatest for the LFRS+AB group, with significantly greater amounts of total bacteria than the HFRS+AB ($p<0.04$) and negative control ($p<0.004$, LFNRS+NAB) groups. The difference between LFRS+AB and LFRS+NAB approached significance ($p=0.07$). There were no differences among all groups for *Lactobacillus spp.* and *Clostridium Cluster XIVa, b* as log CFU/ml or normalized with total bacterial amounts because the F statistic $p>0.05$; but the F value approached significance ($p=0.054$) for *Lactobacillus spp.* results normalized by total bacteria. The LFRS+AB group had the numerically greatest amount of *Bacteroides spp.* as log CFU/ml or normalized with total bacterial amounts, and was significantly different from the negative control group (Log CFU/ml, $p<0.0008$, normalized, $p<0.004$). Also, the positive control group (LFRS+NAB) had greater (Log CFU/ml, $p<0.01$; normalized, $p<0.002$) amounts of *Bacteroides spp.* than the negative control group, but the HFRS+AB group only had greater ($p<0.046$) amounts than the negative control group for the *Bacteroides spp.* as log CFU/ml. The positive control group (LFRS+NAB) had the numerically greatest amounts of *Bifidobacterium spp.* as log CFU/ml, but the negative control (LFNRS+NAB) had the numerically greatest amounts after normalization, likely the result of having the lowest numerical amounts of total bacteria. The LFRS+AB group had significantly lower amounts of *Bifidobacterium spp.* as log CFU/ml or normalized compared to the two control groups (log₁₀ CFU/ml or normalized LFRS+NAB vs. LFRS+AB $p<0.006$ or 0.005 , respectively; log₁₀ CFU/ml or normalized LFNRS+NAB vs. LFRS+AB $p<0.007$ or $p<0.003$, respectively).

4. Discussion

The main result of the second study was a successful knock down of fermentation of RS for use in mechanistic rodent studies to determine if there is any effect of RS on health when there is minimal fermentation. The result may allow future assignment of cause and effect for dietary RS beyond merely an association. This successful knockdown of fermentation occurred when the antibiotics were given simultaneously with the feeding of RS. The initial study 1 investigated treatment with antibiotics prior to feeding RS. However, this study was stopped after we observed robust fermentation in both groups of rats that first received antibiotic treatment, and then were given either a gavage of water or a gavage of cecal contents from rats previously fed RS without prior antibiotic treatment. No development of non-responders occurred.

Although we observed successful knock down of RS fermentation, we also observed several side effects of the antibiotic treatment. These side effects included increases in two variables that are often used as signs of fermentation, ECW [1, 3], and GLP-1 [1, 2, 29], thereby mimicking increased fermentation despite the successful knock down. Another study that used an antibiotic treatment reported hyperglycemia in rats treated with the antibiotic gatifloxacin with over-secretion of GLP-1 and suppressed insulin secretion [30]. Wichmann et al. also demonstrated increased GLP-1 with bacitracin, neomycin, and streptomycin treatment [31].

We also observed no reduction in total bacteria and *Bacteroides spp.*, but a significant decrease in *Bifidobacteriaspp.* for LFRS+AB compared to LFRS+NAB. Our results were similar to those of Cani et al. as their control C57bl6/J mice treated with the same antibiotics had decreased *Bifidobacteria spp.* and increased *Bacteroides spp.* compared to the same type of mice without antibiotics [7]. However, they did not report total bacteria amounts nor normalize bacterial types with total bacteria amounts. The LFRS+AB group had numerically greater amounts of total bacteria than the LFRS+NAB with or without normalization. Unlike Cani et al. [7] we did not observe a statistically significant decrease in *Lactobacillus spp.* which may be a result of their use of C57bl6/J and ob/ob mice compared to our use of Sprague Dawley rats. Thus, the results of the two studies generally indicate reduction in gram-positive bacteria, the target of ampicillin [9], and an increase in gram-negative bacteria. The latter is surprising because neomycin as an aminoglycoside antibiotic is reported to kill gram-negative bacteria [10]. Since both studies using this same antibiotic cocktail in drinking water observed fairly similar results, it is possible that the concentration for neomycin that targets gram negative bacteria that includes genus *Bacteroides* [11] at 0.5 g/L is too low to be effective. Alternatively, our results may indicate that several other more focused antibiotics would need to be added to more thoroughly knockdown total bacteria.

Histological examination (study 2) showed that antibiotic treatment numerically increased the muscularis layer to increase overall height of the cecal wall. This appears to be the reason why the ECWs in study 2 were not significantly different between the LFRS+AB and positive control LFRS+NAB groups. Another study has demonstrated death of mice and rats with very long-term treatment with the antibiotic clinafloxacin as a result of cecal dilatation (abnormal enlargement and stretching) and cecal torsion ending with cecal rupture [32]. This demonstrates that the long-term impact of treatments with antibiotics results in increasingly greater degrees of cecal and other abnormalities [5].

Other evidence of inhibition of fermentation of RS was the doubling of the amount of RS in the cecal contents of rats treated with antibiotics compared to the positive control. Previously we reported the metabolizable energy of the RS product [33]. Subsequently, we estimated that use of mechanistic, proof-of-concept amounts of resistant starch used in our studies were ~50% fermented. This was confirmed as the antibiotic treatment resulted in ~two times the amount of RS in cecal contents.

5. Concluding Remarks

In conclusion, we report a successful knockdown of fermentation of RS in rats using ampicillin and neomycin in drinking water if the antibiotic treatment occurs simultaneously with the feeding of RS. This was not observed if RS is fed to rats after stopping the antibiotic treatment. This will allow for proceeding beyond association of beneficial health effects with fermentation of RS to determination of cause and effect. However, use of antibiotics in the current report and in past reports has been associated with possible confounding side effects. These have also included modulation of the gut microbiota by producing drastic short and long term alterations [34, 35]. One possible solution may be to use antibiotics only for a few days as increased time of antibiotic treatment has resulted in very severe cecal damage [32] compared to only thickening of the cecal wall in our current study. These studies would focus on more short-term effects such as fermentation due to changes in the microbiota, but not longer-term effects such as reduction of body fat accretion. The other possibilities are to either explore other antibiotics and different doses or to seek out other alternatives to antibiotics for inhibition of fermentation of RS or even for reduction of the microbiota for a transplant of cecal contents to improve the microbiota. One possibility for inhibition of fermentation is the use of germ free rodents, but this model is also not free of confounding variables as germ free mice are reported to also have elevated serum levels of GLP-1 [31] and have an immature immune system [36].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

RS	resistant starch
GLP-1	glucagon-like peptide 1
SCFA	short-chain fatty acids
spp	species
AB	antibiotics
NAB	no antibiotics
WtG	water gavage
CG	cecal contents gavage
LFRS	low-fat resistant starch group

HFRS	high-fat resistant starch group
NRS	no resistant starch
ABF%	abdominal fat percent
CFU	colony forming units
ECW	empty cecum weight
AIN-93M	American Institute of Nutrition 1993 mature rodent diet
qPCR	quantitative polymerase chain reaction

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Table 1Diet composition in Study 2.^a

Ingredients	LFRS		HFRS		LFNRS	
	Grams	kcal	Grams	kcal	Grams	kcal
Waxy corn starch ^b	138.4	489.3	0.0	0.0	536.8	1899
High-amylose corn starch ^{c,d}	524.0	1467.2	524.7	1469.1	0.0	0.0
Sucrose	100.0	387.0	100.0	387.0	100.0	387.0
Casein ^e	132.3	473.5	133.7	478.7	136.0	486.9
Cellulose	18.3	0.0	0.0	0.0	135.0	0.0
Soy bean oil ^f	38.9	343.6	93.4	826.0	44.0	388.9
Lard ^g	0.0	0.0	100.0	900.0	0.0	0.0
Mineral mix	35.0	29.4	35.0	29.4	35.0	29.4
Vitamin mix	10.0	38.7	10.0	38.7	10.0	38.7
Choline chloride	1.4	0.0	1.4	0.0	1.4	0.0
L-Cystine	1.8	7.2	1.8	7.2	1.8	7.2
Total	1000	3236	1000	4136	1000	3237

^aDiets include: LFRS = low fat high resistant starch (RS) diet; HFRS = high fat and high RS diet; LFNRS = low fat and amylopectin control corn starch with no resistant starch. Diets with high RS - 23.5% RS by weight of diet.

^b AMIOCA® corn starch is waxy corn starch.

^c HI-MAIZE® resistant corn starch.

^d Both AMIOCA and HI-Maize were gifts from Ingredion Incorporated (Bridgewater, NJ).

^e Casein was reduced from AIN-93M amount (140 g/kg) for diets based on protein in AMIOCA corn starch and HI-MAIZE® resistant corn starch as analyzed by proximate analysis by Medallion Labs.

^f Soybean oil was adjusted from AIN-93M amount (40 g/kg) for LFRS and HFRS (100 g/kg) diets based on fat in the AMIOCA and HI-MAIZE as analyzed by proximate analysis by Medallion Labs. It was also increased to manipulate energy value for LFNRS diet.

^g Lard was added in combination with soybean oil for producing ~42% of energy as fat for HFRS diet.

Table 2

Cecal contents pH, SCFAs, Resistant starch (RS), and Empty Cecum Weight (ECW), serum active glucagon-like peptide 1 (GLP-1), and abdominal fat percent (ABF%) for study 2^{a,b}.

Variables	LFRS+AB	HFRS+AB	LFNRS+NAB	LFRS+NAB	Pooled SEM
pH	8.23 ¹	8.39 ¹	8.39 ¹	6.05 ²	0.12
ECW ⁵ (g)	1.27 ^{1,2}	1.08 ²	0.51 ³	1.45 ¹	0.09
Acetate (mmol)	0.151b	0.149bc	0.060c	0.469a	0.04
Propionate (mmol)	0.096 ¹	0.072 ²	0.013 ³	0.087 ^{1,2}	0.017
Butyrate (mmol)	0.004 ¹	0.004 ¹	0.013 ²	0.074 ³	0.003
ABF% ⁶	1.46 ¹	1.81 ²	1.41 ¹	1.38 ¹	0.12
RS (g/100 g)	19.4 ¹	20.1 ¹	0.07 ²	10.1 ³	0.64
GLP-1 active (pM)	1.64 ^{1,2}	1.53 ¹	0.24 ³	0.85 ²	0.19

^aData are presented in their original form and expressed as means ± pooled standard error. P value <0.05 was considered statistically significant and is represented with different numbers across rows.

^bGroups include: LFRS+AB = low fat, high RS diet + antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L in drinking water); HFRS+AB = high fat, high RS diet + antibiotic treatment; LFNRS+NAB = low fat, no RS diet + no antibiotic treatment; LFRS+NAB = low fat high RS diet + no antibiotic treatment.

Table 3

Study 2 Bacterial qPCR^{a,b}

Bacterial Group	Log CFU/ml ^c or $\left(\frac{\text{Bacterial Group}}{\text{Total Bacteria}}\right)^d$					p-value (F) ^e
	LFRS+ AB	HFRS+AB	LFRS +NAB ^c	LFNRS +NAB ^d	Pooled SEM	
Bacterial Domain						
Total Bacteria	9.2678 ¹ (−)	8.8290 ² (−)	8.8862 ^{1,2} (−)	8.5838 ² (−)	0.0645 (−)	0.0334
<i>Escherichia coli</i>						
Firmicutes						
<i>Lactobacillus spp</i>	2.4579 (−6.8099)	2.3879 (−6.4411)	3.2528 (−5.6333)	3.6067 (−4.9771)	0.1910 (0.2317)	0.1260 (0.0541)
Clostridium cluster XIVa and XIVb	6.3845 (−2.8833)	5.9267 (−2.9023)	6.6911 (−2.1950)	6.7660 (−1.8178)	0.2188 (0.2381)	0.5333 (0.1564)
Bacteroidetes						
Bacteroides group including <i>Prevotella</i> and <i>Porphyromonas</i>	6.9055 ¹ (−2.3623 ¹)	5.6351 ¹ (−3.1940 ^{1,2})	6.0901 ¹ (−2.7961 ¹)	4.2661 ² (−4.3577 ²)	0.2305 (0.2106)	0.0064 (0.0238)
Actinobacteria						
<i>Bifidobacterium spp</i>	0.7743 ¹ (−8.4936 ¹)	1.5138 ¹ (−7.3718 ^{1,2})	3.4761 ² (−5.4100 ^{2,3})	3.4139 ² (−5.1698 ³)	0.3047 (0.3403)	0.0117 (0.0080)

^a Groups include: LFRS+AB = low fat, high RS diet + antibiotic treatment (Ampicillin 1g/L and Neomycin 0.5g/L in drinking water); HFRS+AB = high fat, high RS diet + antibiotic treatment; LFNRS+NAB = low fat, no RS diet + no antibiotic treatment; LFRS+NAB = low fat, high RS diet + no antibiotic treatment.

^b A p<0.05 was considered statistically significant and different numerical superscripts across rows indicate differences among treatment groups.

^c The upper number for each treatment group per bacterial group is log CFU/ml.

^d The bottom number for each treatment group per bacterial group is normalized by total bacteria; and since both numbers are log base 10 a subtraction is performed. Also note that the pooled SEM is not a log, but represents the absolute variance.

^e A F-ANOVA statistic (p<0.05) indicates a significant measurement. No superscript numbers across rows occurs if the F-ANOVA statistic was not significant.