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In Vivo Colonic Fermentation of Sorghum (*Sorghum bicolor* L.): Important Correlations Observed among the Physiological Parameters of Cecum, Liver, Adipose Tissue and Fasting Serum Lipid Profile

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Summary High amylose corn starch (HAS), whole grain sorghum (S-Wh), refined sorghum (S-Rf) and α-corn starch (CON) diets were fed to animals for 1 mo aiming to examine the physiological effects of resistant starch inclusion in the diet from grains. HAS exhibited significantly lower feed intake, final body weight, serum lipid profile with significantly higher cecal parameters and short chain fatty acid (SCFA) contents. S-Wh group exhibited significantly higher body weight, feed intake and serum lipid parameters compared to other 3 groups. Cecal fermentation was not seemed to be prominent in the CON, S-Wh and S-Rf groups with respect to lower cecal parameters and SCFA contents. The cecal microbial compositions in HAS, S-Wh and CON/S-Rf exhibited 3 distinct clusters suggesting a significant effect of the cecal microbial composition on cecal parameters, SCFA contents and physiological parameters. .

Key Words resistant starch, serum lipids, correlations, whole grain, SCFA

Non-digestible carbohydrates (NCs) such as resistant starch (RS) are known to possess beneficial physiological and biological effects, such as weight management, reduction of calorie intake, glucose homeostasis and lipid metabolism (*1*). These beneficial health effects are attributed to the gut microbial fermentation metabolites, mainly short chain fatty acids (SCFA) (*1*).

Sorghum (*Sorghum bicolor* L.), is an indigenous cereal crop in Africa with unique nutritional properties, despite of underutilization as a human food source. Low in vitro flour digestibility of sorghum, is considered as a potential trait for overweight and obesity management, and is found to be associated with low digestibility of both starch and protein fractions (*2*). With the low digestibility of starch, it becomes an assuring source of RS with an average between 12.0–21.5%, presenting potentials as a prebiotic substrate (*2*).

Natural RS food sources such as minor cereals, have earned the spotlight of many studies recently, aiming to mitigate escalating increase of chronic metabolic diseases directly associated with diet, for instance co-morbidities that encompasses metabolic syndrome. Thus the aim of this study was to evaluate the physiological effects of gut fermentation of RS from grain sources in comparison to a well-characterized RS source, high amylose starch (HAS).

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Materials and Methods

Experimental diets. Two types of sorghum flour, whole sorghum (S-Wh) and refined sorghum (S-Rf) flour were provided by Nakano Industry Corporation (Takamatsu, Japan) and the experimental diets (30% w/w) substitution by S-Wh, S-Rf and HAS sources instead of α-corn starch in control (CON) diet were prepared according to AIN-93G guidelines by Oriental Yeast Co., Ltd., (Tokyo, Japan).

Animal experimental design, care for laboratory animals and post-mortem excision of organs. Twenty four F344 male rats (7 wk old; average body weight 130–160 g) were purchased from Charles River Japan (Yokohama, Japan). After acclimatization for one week, rats were devided into four similar body weight groups (6 rats/group) and the feeding experiment was conducted for four weeks. The maintenance of animals, daily and weekly routine measurements, sacrifice, post mortem organ excision and handling were conducted according to the methods described in (*3*). The animal experiment was conducted according to the guidelines of "Guide for the Care and Use of Laboratory Animals" and all the procedures were approved by the Animal Care and Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine (License no: 29–94).

Rat cecal bacterial DNA extraction, sequencing and analysis of 16S rRNA sequences. Bacterial DNA was extracted from the cecal digesta employing the modified phenol-free repeated beads beating plus column (RBB+C) method described in (*4*). Extracted genomic DNA was purified via sequential digestions with RNase

Table 1. Feed intake, body weight parameters and internal organ parameters of rats fed α-corn starch (CON), high amylose starch (HAS), whole sorghum (S-Wh) and refined sorghum (S-Rf) flour diets.

Parameter	Feed Groups							
	CON		HAS		S-Wh		S-Rf	
FBW (g)	243±3	ab	232±3	b	246±3	a	244±3	ab
BWG (g)	69±2	a	57±2	b	71±3	a	68±1	a
FI (g)	380±5	ab	365±5	b	393±5	a	381±5	ab
Liver weight (g)	9.43±0.16	a	8.32±0.12	bc	9±0.16	ab	8.26±0.24	c
Adipose tissue weight								
Perirenal (g)	4.53±0.12	ab	3.75±0.25	b	4.86±0.3	a	4.76±0.16	a
Epidydimal (g)	5.29±0.16	a	4.09±0.19	b	5.41±0.38	a	5.23±0.17	a
Cecum								
CW (g)	2.53±0.28	b	5.82±0.71	a	2.18±0.09	b	1.96±0.21	b
CTW (g)	0.55±0.03	b	0.99±0.06	a	0.5±0.02	b	0.51±0.02	b
CCW (g)	1.98±0.29	b	4.83±0.68	a	1.68±0.08	b	1.46±0.22	b
pH	7.11±0.11	a	6.32±0.11	b	6.86±0.04	a	6.9±0.05	a
Ammonia-N (mg/g cecum)	0.84±0.09	a	0.46±0.07	b	1.11±0.07	a	1.07±0.06	a
IgA (μg/g cecum)	99±5	ns	163±23	ns	110±4	ns	117±22	ns
SCFA (μmol/g cecum)								
Acetate	80.46±11.44	*ab*	115.3±15.47	*a*	83.54±5.95	*ab*	74.8±9.93	*b*
Propionate	13.05±1.40	ns	11.33±1.52	ns	9.37±0.88	ns	10.71±1.51	ns
Butyrate	3.47±0.58	b	9.83±1.37	a	5.30±0.62	b	3.46±1.29	b
Total SCFA	96.98±13.25	b	136.45±17.43	a	98.21±6.32	b	88.95±12.03	b

Abrev: FBW; final body weight, BWG; body weight gain, FI; feed intake, CW; cecal weight, CTW; cecal tissue weight, CCW; cecal content weight, IgA; immunoglobulin-A, SCFA; short chain fatty acid, ns; not statistically significant.
Mean values±SE are presented in the table; a, b and c in each row represent significant differences at $p<0.05$ respectively. *a, b* represent significant differences at $p<0.1$.

and proteinase K (QIAGEN, Valencia, California, United States).

V3 and V4 variable regions of 16S rRNA were amplified using bacterial overhang adapters and universal primers in the first stage PCR; forward primer (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3′) and the reverse primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GATTACHVGGGTATCTAATCC-3′). Second stage PCR, sequencing of PCR products and the analysis of retrieved raw 16S rRNA gene sequences was conducted according to the method reported in (5). The generated biome table was normalized using an equal subsampling size of 11667 sequences. Distances between bacterial communities in different samples were calculated by the weighted UniFrac distance metric and Principle Coordinate Analysis (PCoA) plots generated in QIIME. Calypso version 8.72 was used to generate Least Discriminant Analysis effect size (LEfSe) plot.

Rat cecal SCFA analysis by high performance liquid chromatography (HPLC). SCFA content in diluted cecal digesta of rats were analyzed by HPLC (Shimadzu LC-10AD, Kyoto, Japan). Samples were prepared according to the method described in (*3*) and analytical specifications were as follows; column, RSpak KC-811 (8.0 mm×300 mm, Shodex, Tokyo, Japan); eluent and flow rate, 2 mM $HClO_4$ at 1 mL/min; column temperature, 47˚C; reaction reagent and flow rate, ST3-R (×10 diluted) at 0.5 mL/min; UV detector wavelength, 450 nm.

Ammonia-nitrogen (AN), Immunoglobulin A (IgA) in cecal digesta and serum (fasting) biochemical analysis. AN and IgA in the diluted samples of cecal content and the serum biochemical parameters were analyzed according to the procedures reported in (*6*).

Statistical analysis. All data were analyzed for their significance ($p<0.05$) by analysis of variance (ANOVA) using SPSS statistical software version 17.0 (IBM Co., Armonk, New York, United States) coupled with Tukey's test. Correlations among the parameters were obtained by Pearson's correlation analysis tool in SPSS.

Results

At the end of the experimental period final body weight (FBW) was significantly ($p<0.05$) lower in the HAS group compared to S-Wh group (Table I). FI and FBW had a similar trend, as body weight gain (BWG) was significantly ($p<0.05$) lower in the HAS group compared to the other three groups. Perirenal (Pe-AT) and epididymal adipose tissue (Ep-AT) weights were significantly ($p<0.05$) lower in the HAS group in comparison to the two sorghum groups. Liver weight was significantly ($p<0.05$) lower in the S-Rf group compared to the CON group.

Cecal parameters were significantly ($p<0.05$) higher in the HAS group while other three groups reported significantly ($p<0.05$) lower values. Cecal pH was significantly ($p<0.05$) lower in the HAS group compared to

Table 2. Pearson's correlation coefficients among the physiological and serum lipid parameters.

Parameter	BWG	FI	FBW	Liver	Pe-AT	Ep-AT	CW	CTW	CCW	Ac	Pr	Bu	TSCFA	pH	SI	OBI	IgA	AN	TC	HDL-C	non-HDL-C	C-ratio	TG	NEFA
FI	.552**																							
FBW	.691**	.869**																						
Liver	.474*	.543**	.599**																					
Pe-AT	.645**	.733**	.614**	0.254																				
Ep-AT	.693**	.703**	.671**	.427*	.832**																			
CW	−.607**	−.458*	−.546**	−0.264	−.510*	−.544**																		
CTW	−.691**	−.449*	−.488*	−0.257	−.551**	−.605**	.868**																	
CCW	−.583**	−.450*	−.542**	−0.259	−.494*	−.524**	.998**	.832**																
Ac	−0.208	−0.403	−.445*	−0.179	−0.281	−0.258	.576**	.416*	.586**															
Pr	−0.082	−.508*	−0.314	−0.013	−0.286	−0.149	0.059	−0.047	0.073	.477*														
Bu	−.561**	−.566**	−.686**	−0.317	−.500*	−.630**	.675**	.640**	.666**	.482*	0.202													
TSCFA	−0.247	−.463*	−.494*	−0.193	−0.326	−0.305	.591**	.433*	.600**	.994**	.539**	.547**												
pH	.568**	.555**	.635**	.508*	.469*	.574**	−.854**	−.723**	−.854**	−.507*	−0.053	−.720**	−.532**											
SI	0.124	0.233	0.338	0.244	0.226	0.296	−0.110	−0.079	−0.113	−0.283	0.133	−0.409	−0.283	0.392										
OBI	.584**	.543**	.638**	0.340	.588**	.653**	−.628**	−.566**	−.624**	−.552**	−0.067	−.655**	−.567**	.700**	.737**									
IgA	−0.387	−0.035	−0.219	−0.331	−0.195	−0.329	.622**	.439*	.635**	0.326	−0.099	0.305	0.315	−.487*	0.152	−0.247								
AN	−0.305	−0.357	−.499*	−0.151	−0.277	−0.204	.614**	0.269	.649**	.537**	0.300	.542**	.564**	−.588**	−0.243	−.481*	.483*							
TC	.563**	.620**	.584**	.573**	.581**	.750**	−.442*	−.514*	−.423*	−0.113	0.000	−.428*	−0.142	.492*	0.383	.587**	−0.293	−0.148						
HDL-C	0.268	.600**	.422*	0.090	.518**	.492*	−0.193	−0.190	−0.189	−0.020	−0.335	−0.106	−0.057	0.003	−0.113	0.188	0.007	−0.052	.608**					
non-HDL-C	.569**	.530**	.544**	.636**	.511*	.715**	−.452*	−.536**	−.431*	−0.125	0.106	−.463*	−0.146	.569**	.478*	.620**	−0.342	−0.155	.968**	0.389				
C-ratio	0.356	0.052	0.200	.578**	0.106	0.303	−0.289	−0.378	−0.271	−0.103	0.358	−0.371	−0.097	.563**	.524*	.434*	−0.352	−0.108	.432*	−.450*	.643**			
TG	.650**	.518**	.503*	.415*	.654**	.760**	−.572**	−.584**	−.558**	−0.209	−0.089	−0.360	−0.231	.445*	0.105	.589**	−.471*	−0.209	.751**	.514*	.709**	0.271		
NEFA	0.287	0.159	0.179	.451*	0.346	.432*	−0.307	−.451*	−0.281	−0.101	0.210	−0.277	−0.099	0.353	0.246	0.325	−0.327	0.162	.520**	−0.032	.613**	.646**	.519**	
Glucose	.483*	0.302	0.267	0.317	.484*	.530**	−0.371	−.464*	−0.350	−0.085	0.054	−0.312	−0.101	.502*	0.289	0.381	−0.217	−0.230	.417*	−0.092	.512*	.606**	0.381	.436*

Abbrev: BWG; body weight gain, FBW; final body weight, FI; feed intake, Pe-AT; perirenal adipose tissue, Ep-AT; epididymal adipose tissue, CW; cecal weight, CTW; cecal tissue weight, CCW; cecal content weight, Ac; acetic acid, Pr; propionic acid, Bu; butyric acid, TSCFA; total SCFA, SI; Shannon index, OBI; observed species index, IgA; Imuunoglobulin A, AN; ammonia-nitrogen, TC; total cholesterol, HDL-C; HDL cholesterol, non-HDL-C; non-HDL cholesterol, C-ratio; cholesterol ratio, TG; triglycerides, NEFA; non-esterified fatty acids,; * $p<0.05$, ** $p<0.01$.

other three groups. AN content in the HAS fed group was significantly ($p<0.05$) lower compared to the other three groups. Acetic acid, butyric acid and total SCFA contents followed similar trends where, HAS fed group reported significantly ($p<0.05$) higher contents while other three groups had similar lower contents.

Total cholesterol (TC), HDL cholesterol (HDL-C), triglycerides (TG) in the serum at the end of the experimental period were significantly ($p<0.05$) higher in the S-Wh and the CON fed groups in comparison to HAS fed group (data not shown). Non-HDL cholesterol (non-HDL-C) and glucose contents were significantly ($p<0.05$) higher in the CON and the S-Wh fed groups. Non-esterified fatty acid (NEFA) content in the HAS group was significantly ($p<0.05$) lower than the CON.

Discussion

FBW and BWG at the end of the experimental period in S-Wh, S-Rf and CON groups, seemed to be significantly contributed by the liver weight, Pe-AT weight and Ep-AT weight as further backed by the positive correlation coefficients, suggesting an effect of adiposity (Table 2). The direct contributions to FBW and BWG, from liver weight, adipose tissue weights in the previously mentioned groups were further illustrated by the Pearson's correlation coefficients. On the other hand, cecal fermentation might have negatively affected FBW, BWG and other organ weights, which might have been exhibited by the negative correlations with CW, CTW and CCW, an observation clearly apparent in the HAS group (*7*). Albeit, the colonic fermentation effect was not much clear in the raw data, Pearson's correlation analysis provided solid evidences for the fermentation effects by the negative correlation values for cecal parameters and SCFA with body weight parameters, adipose tissue weights and FI (*7*). The correlation effects indicated that cecal SCFA, especially propionic acid content significantly reduced FI which might have been due to its pronounced satiety inducing effect (*8*). Further, butyric acid and total SCFA contents seemed to have negatively affected FI resulting lower FBW and BWG, possibly which might have been due to the lower adipose tissue weights which was apparent in HAS, and it was further evinced by the negative correlations between butyric content and adipose tissue weights. Acetic acid seemed to have affected FBW which might have been due to the effects of other effector molecules either upregulated or downregulated by acetic acid (*9*). Higher RS content in HAS (30% w/w dry weight basis) might have been a good fermentative substrates for the key bacterial species identified in the HAS group; *Bifidobacterium, Ruminococcus, Bacteroides* and *Parabacteroides* (data not shown) who are known for their complex carbohydrate degrading ability in the colon (*10*). Similar acetic acid level observed in CON group with S-Wh can be attributed to its higher relative abundance of genus *Blautia*, identified as an acetogenic microorganism closely associated with mucus layer, even though the CON diet was deficient in fermentable carbohydrates (*10*).

As suggested by the correlations, significantly higher FI might have been the culprit for higher FBW, BWG, adipose tissue weights and liver weights along with higher serum TC, HDL-C and non-HDL-C levels in the S-Wh group (Table 2). Albeit, the cecal propionic acid content was not significantly different among the groups, relative differences might have played a suppressing effects (i.e. releasing PYY, GLP-1) on FI as manifested by the negative correlation coefficient (*11*).

Negative correlations for CW, CTW and CCW and positive correlations for cecal pH with TC, non-HDL-C and TG in serum, further indicated anti-lipidemic effects of cecal fermentation (*12*). Interestingly, only cecal butyric acid content exhibited a significant negative correlation with serum TC and non-HDL-C. This might have been an indirect manifest of the trophic effect of butyric acid on cecal tissue proliferation or an effect of activation of GPR109A receptor in adipose tissue or hepatocytes, which responds only to butyric acid (*9*, *13*). The correlations between serum lipid profile and cecal butyrate content could be identified as a cross-sectional view of the scenario in the adipose tissue masses, as they exhibited the same trend.

The contribution to serum TC from non-HDL-C seemed to be more prominent over HDL-C, according to Pearson's coefficients among the above parameters and cholesterol ratio, which was evident in CON and S-Wh groups. Cholesterol ratio is considered as a better indicator of risk of heart diseases, and it indicated that HAS, S-Wh and S-Rf posed significantly lower risk compared to CON (*14*). Serum TG can be originated either from dietary fats (form of chylomicrons) or hepatic de novo lipogenesis (form of very low density lipoproteins) (*15*). Thus, the similar trend in correlations observed for serum non-HDL-C and TG with FI, liver and adipose tissue weights could be either due to higher dietary fat intake and/or higher hepatic lipogenesis. Yet, considering the magnitude of the correlations for TG with FI and non-HDL-C in the S-Wh group, the latter seemed to have caused a notable effect, suggesting a higher hepatic lipogenesis.

Serum NEFA content is determined by the balance among lipolysis in adipose tissues and fatty acid oxidation in muscles and de novo lipogenesis in liver, where a higher NEFA content in the serum might suggest an abnormal lipolysis in adipose tissues or impaired ability of liver to export lipogenesis products or utilize NEFA (*12*, *16*). Relationships of serum NEFA with Ep-AT and liver weights might suggest an increased lipolysis in Ep-AT depot and higher NEFA uptake and utilization by liver, as evident in CON group.

A higher NEFA content persisting in serum causes peripheral insulin resistance due to the competition between NEFA and glucose as the energy source for muscles and liver leading to type II diabetes as a result of damaged pancreatic β-cells (*16*). Insulin resistance can be caused by abnormal adipocyte function (due to inflammation or hypertrophy) manifesting an impaired sensitivity of hormone sensitive lipase to insulin mediated dephosphorylating activity (*16*). This can be caused by either genetic (genetically pre-disposed to type

II diabetes) or physiological factors (metabolic disorders, unhealthy living habits) resulting a flux of NEFA due to uncontrolled lipolysis or inability to neutralize and store NEFA (*16*). On the other hand, impaired hepatocyte function again which could be due to genetic, neurohumoral, metabolic or stress-related factors (i.e. non-alcoholic fatty liver disease) might cause impairments in β-oxidation of NEFA or TG synthesis and export resulting lower uptake by liver, which results in impaired glucose tolerance in body increasing serum glucose levels (*16*). Higher serum glucose levels and their correlations with FI and adipose tissue weights in CON and S-Wh groups might reveal the contributing counterparts, while Ep-AT weight showed a higher effect suggesting impaired adipocyte functions, which might have been due to the increased adipocyte size (*16*).

Observed species index is an indicator of α-diversity which provides information about the number of different species observed in the particular group. Interestingly, the negative correlations with cecal parameters and SCFA and positive correlations with serum lipid parameters, FI, FBW, BWG and adipose tissue weights might suggest that the majority of the species observed, especially in the CON group might have favored lipogenesis. Lack of a correlation with cecal propionic acid content might suggest the lack of species expressing propionic acid producing enzymes, which was elucidated by the raw data itself too.

On the other hand, Shannon index, the indicator of species relative abundance and evenness was not much correlated as the observed species index. Lack of correlations with cecal parameters and SCFA might suggest the negligible contribution to cecal fermentation by the bacterial OTUs observed in higher abundance in the CON and S-Rf groups. These correlations of α-diversity indices with cecal and SCFA parameters might explain the observed lower SCFA contents in the mentioned groups. Further, the positive correlations for Shannon index with non-HDL-C and cholesterol ratio might suggest the higher relative abundance of bacterial OTUs that might have favored atherogenic cholesterol and TC increment in serum, which was clearly unraveled in the raw data for CON, S-Rf and S-Wh groups. Thus, the chracteristic bacterial genera observed in CON, S-Rf and S-Wh (data not shown) might have been associated with obesity or hyperlipidemic phenotypes.

Origin of cecal ammonia can be either by urea hydrolysis or amino acid fermentation (*17*). According to the correlations observed between ammonia content and CW and CCW, it is obvious that the origin might have been amino acid fermentation by microbiota. Further, the relationships between ammonia and cecal acetic acid, butyric acid and total SCFA contents might suggest that amino acid fermentation in the cecum might have contributed to their concentrations in S-Wh, S-Rf and CON groups (*18*). The resistant nature of protein (Kafirin; a prolamin) in the S-Wh and S-Rf groups as previously reported and lack of fermentable carbohydrate substrates in CON group might have caused amino acid fermentation and higher cecal ammonia content (*18*). Generally, cecal pH is considered to be increased in the presence of a higher ammonia concentration which is suggested by the observed correlation between pH and ammonia content (*19*). Ammonia is considered as a cytotoxic and its higher concentrations are known to cause stress in animals, hindering their growth as suggested by the negative correlations between ammonia and FBW. Yet, the negative correlation between observed species index and cecal ammonia content might suggest that the higher number of different observed species in CON might not have been associated with ammonia production, while a majority of different species observed in the two sorghum groups might have been associated as manifested by the cecal ammonia contents.

The positive correlation observed between IgA and cecal parameters might explain the observations for cecal parameters and IgA expression in the HAS group. Lack of a correlation with the observed species index and Shannon index might explain the similar IgA expressions across the four diet groups, which was also apparently observed in the clustered bar chart for genera distribution (figure not shown) among the four groups with higher intra-group variabilities that might have caused higher standard error in HAS. Further, the positive relationship with ammonia might have suggested a link between ammonia producing OTUs and IgA expression.

Disclosure at State of COI

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