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Evaluation of fermentation profiles of starches from different plant sources in an *in vitro* batch culture

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Target audience: *Monogastric nutritionists and researchers, research and development (R&D)*

Abstract

The influence of starches containing different levels of resistant starch (RS) on short chain fatty acid formation after fermentation was investigated in an in vitro batch culture. Native starches of sago, sweet potato, potato, arrowroot, rice, wheat, corn, as well as tapioca, cassava pulp and sweet potato root meal were evaluated in buffered caecal inoculum of 28-day old broiler chicks using the cumulative gas production technique. Total starch (TS), resistant starch (RS), short chain fatty acids: acetic, propionic and butyric acids. Short chain fatty acid ratios and fermentation ratios were estimated. Total and resistant starch content of the test starches and their short chain fatty acid profile: acetic, propionic and butyric acids- varied ($p<0.05$) amongst test starches. There was a strong relationship observed between proportions of acetic, butyric and propionic acids and total short chain fatty acids with R^2 values ranging from 0.97 to 0.99, However a weak relationship exist between proportions of acetic, butyric and propionic acids and resistant starch contents of the starches with R^2 ranging from 0.19 to 0.22, highlighting that variations in short chain fatty acid profiles of the fermented starches investigated in vitro was due to plant source rather than RS content of the test starches.

Keywords: *Native starch; Resistant starch; Short chain fatty acids; Fermentation ratios; Caecal inoculum*

Description of the problem

The quest for alternatives to antibiotics for non-ruminant production has identified the manipulation of dietary components of feeds as a safer and more environmentally friendly option (1), hence the focus of research on non-digestible carbohydrates such as resistant starch (RS). Resistant starch is the total amount of starch and the products of starch degradation that escape digestion in the small intestine (2). The influence of RS on maintaining gut microbiota profile dominated by health promoting bacteria at the expense of pathogenic bacteria has ignited interest in the fermentative fate of starches of different botanical origin (3-5).

Extensive bacterial fermentation of non-digestible carbohydrates in the hindgut of non-ruminants result in the formation of short chain fatty acids, especially acetic acid, propionic acid and butyric acid, which account for 90 to 95% of the total fatty acids produced in the hindgut (6), methane, hydrogen, carbon dioxide and ammonia (7). The short chain fatty acids formed (butyric acid in particular) are generally considered to confer beneficial physiological effects on the host which include reducing intestinal pH, lowering the production of harmful fermentation by-products such as secondary bile acids, ammonia, phenols (8) and preventing the

degradation of the mucous layer within the colon (9).

The type and level of non-digestible carbohydrates, chemical structure of the constituent polysaccharides, activities of the intestinal microbial population and gastrointestinal tract transit time (10-11) are believed to control the production and molar distribution of short chain fatty acids, with the fermentation of starches furnishing high proportions of butyric acid *in vitro* compared to other non-digestible carbohydrates (11).

Most scientific research on the fermentative qualities of resistant starch, have focused on the RS2 high amylose maize starch (HAMS) source with other forms of resistant starch less researched (12). Hence, this study was initiated to evaluate native starches obtained from cereal and tuber origins for their fermentability in buffered caecal inoculum using the cumulative gas production technique.

Materials and Methods

Sample Preparation and Chemical Analysis

Native starches of sago, sweet potato, potato, arrowroot, rice, wheat, corn and tapioca (cassava starch pearls), were obtained commercially in New South Wales, Australia. Cassava pulp, a by-product of cassava starch production was prepared by peeling the cassava (*Manihot esculenta*) tuber to remove indelible portions, wet milled to slurry and sieved through a double layer muslin cheese cloth to extract the starch. The portion held back in the cheese cloth was then air-dried and milled to obtain the cassava pulp. Sweet potato root meal was prepared from whole sweet potato (*Ipomea batatas)* roots which were washed to remove adhering contaminants, sliced into 2-5mm chips and air-dried for 72 hours. Thereafter the chips were milled and used in this study. All starches were milled through a 1 mm sieve and their total and resistant starch determined using outlined methods for the Megazyme RS and Total Starch assay kit (Megazyme International Wicklow, Ireland).

Inoculum Preparation and Fermentation Incubations

Fresh caecal contents was collected from euthanized, 28-day old broiler birds grown under organic conditions and fed a standard diet free of antibiotics and copper (13). Caeca was harvested from the broiler birds and caecal content pooled into a beaker, weighed then diluted with sterile saline (9g/l sodium chloride) solution and homogenized in a bag mixer (Interscience, St. Norm, France) for 120 seconds to obtain slurry. The slurry was then filtered through a double layered cheese cloth and the filtrate subjected to slow centrifugation at 150 × *g* for 20 min, 15^{o}C (Induction Drive Centrifugation, Beckman Model J2-21M, Beckman Instruments Inc., Palo Alto, California, USA) to separate large feed particles (14). The supernatant was used as inoculum. Approximately 500mg of each test substrate was incubated in inoculum + anaerobic, nitrogen-free buffer (Table 1 (15)) at 39^{o}C for 102 hr; all incubations were carried out in triplicates. The entire process of inoculum preparation was carried out under the flow of O_2-free CO_2

Post Fermentation Analysis

At the end of the incubations, fermentation vessels were centrifuged and the supernatant analyzed for short-chain fatty acids- acetic, propionic and butyric acids. Short chain fatty acids were determined by gas chromatography (GC, Model CP 3800, Varian Analytical Instruments, Palo Alto, CA, USA). The GC was equipped with a flame ionization detector and a polyethene glycol packed column (0.32mm internal diameter, 30m length and 0.25µm film thickness) (Alltech ECONO-CAP$_{TM}$, Alltech Associations Inc., Deerfield, IL, USA). The column was operated at 70-240^{o}C with high purity helium at 20ml/min as

the carrier gas. Short chain fatty acid ratios and fermentation ratios were estimated for each test substrate and total short chain fatty acid was calculated as the sum of acetic acid + butyric acid + propionic acid (16).

Statistical analysis

Statistical analysis was performed using the one-way analysis of variance (ANOVA) procedure of the SAS statistical program and relationships among variables was quantified with simple linear regression analysis using REG procedure of the same package. The MEANS option of GLM procedure was used to calculate means and errors of the means. Means were separated using the Duncan multiple range test.

Animal Ethics

This study was approved by the Animal Ethics Committee of the University of New England, authority number AEC 09/024. Health and husbandry practices complied with the "Australian code of the care of animals for scientific purposes" issued by the National Health and Medical Research Council.

Results and Discussion

One of the interests in resistant starch as a component of non-ruminant feeds is in its ability to serve as substrate for hindgut fermentation, promoting short chain fatty acid production especially for butyrate and positively impacting on gut health. Total starch, resistant starch, short chain fatty acids (SCFAs) as well as SCFA ratios and fermentation ratios for the test starches are shown in Table 2. Total starch of the test starches varied considerably ($p<0.05$) ranging from 57.51 to 84.81% for sweet potato and sago starch, respectively. Total starch in Sago starch was similar to sweet potato starch, wheat starch and corn starch. Resistant starch contents of the test starches also varied ($p<0.05$) with greater values recorded for potato starch (24.08 %) and least values recorded for wheat starch (0.78 %). Differences among test substrates in total starch, resistant starch, short chain fatty acids, short chain fatty acid ratios and fermentation ratios indicated variations in their chemical compositions and fermentative fates. Total and resistant starch values obtained for the test substrates varied in comparison with values reported by (17), except for rice and sweet potato. Variations in values in comparison with literature despite similar methods of determination employed could be attributed to the sample forms i.e. flours, grains or starches, and processing conditions to which they have been subjected (16-17).

There were differences ($p<0.05$) in acetic, propionic and butyric acid as well as TSCFA produced by test substrates with higher values recorded for rice starch (32.96, 5.54, 19.28 and 57.78 μmol AAE/ml, respectively) and least values for potato starch (10.66, 1.52, 3.87 and 16.05 μmol AAE/ml, respectively). Variations in SCFA profile of fermented starches observed in this study could be attributed to the source and structure of their resistant starch as evidenced in other *in vivo* and *in vitro* studies (4, 18, 19). The SCFA ratios varied significantly ($p<0.05$) for all test starches with maximum and minimum values as follows: acetic acid/TSCFA, 0.66 for potato starch and 0.56 for sweet potato root meal; propionic acid/TSCFA, 0.11 for arrowroot starch and tapioca and 0.09 for wheat starch, potato starch and rice starch and butyric acid/TSCFA, 0.33 for rice starch and sweet potato root meal and 0.24 for potato starch. The fermentation ratios i.e. acetic acid. RS, propionic acid/RS and butyric acid/RS also varied and were greater for wheat starch (29.56, 4.59 and 14.98, respectively) and least for potato starch (0.47, 0.07 and 0.17, respectively).

Across the different starches, a strong relationship was observed between total short chain fatty acid (TSCFA) and proportions of

acetic (R^2 = 0.99), propionic (R^2 = 0.97) and butyric (R^2 = 0.98) acids (Figure 1). On the other hand, a weak relationship existed between RS content of the test starches and proportions of acetic (R^2 = 0.04), propionic (R^2 = 0.02) and butyric (R^2 = 0.04) acids, (i.e. higher resistant starch content of the test substrates did not translate to higher short chain fatty acid production) implying that variations in short chain fatty acid profiles of the fermented starches was due to their source and the structure of their RS rather than RS content of the test starches (Figure 2). On the contrary, analysis of data from an *in vivo* study by (20), showed a positive relationship between resistant starch levels of different resistant starch preparations included in the diets of rats and TSCFA and acetate levels in the caecum. On the other hand, a weak relationship was recorded between resistant starch levels of the different resistant starch preparations and propionate and butyrate levels in the caecum. This indicates a need to corroborate *in vitro* findings in *in vivo* determinations. Despite variations between acetic, propionic and butyric acid produces by the different test substrates, molar ratios (Figure 3) fell within the range of documented molar ratios for starches fermented *in vitro* as reviewed by (11).

Conclusion and Application

1. Short chain fatty acid as fractions of total short chain fatty acids (TSCFA) followed the order acetic acid > butyric acid > propionic acid.
2. Ratio of TSCFA and SCFA to resistant starch in all the test starches followed the order TSCFA > acetic acid > butyric acid > propionic acid.
3. Variability in short chain fatty acid profile, SCFA ratios and fermentation ratios of the starches studied can be attributed to their source and structure rather than their resistant starch content.

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Table 1: Component of nitrogen-free anaerobic medium (Williams et al., 2005)

Component	Concentration in medium
	ml/L
Basal solution	
Resazurin solution [a]	1.00
Haemin solution [b]	10.00
Fatty acid solution [c]	10.00
Distilled water	979.00
	g/l
KCl	0.60
NaCl	0.60
$CaCl_2.2H_2O$	0.20
$MgSO_4.7H_2O$	0.50
KH_2PO_4	1.46
	mL/vessel
Basal solution	72.00
Reducing solution [d]	1.00
Vitamin-phosphate solution [e]	1.00
Bicarbonate solution [f]	4.00

[a] Composition: 0.2 g, resazurin per 200 ml distilled water.
[b] Composition: 500 mg; hemin in 10% sodium hydroxide (NaOH) solution.
[c] Composition : 6.85 ml; acetic acid, 3.00 ml; propionic acid, 1.84 ml; butyric acid, 0.47 ml; *iso*-butyric acid, 0.55 ml; 2-methyl-butyic, 0.55 ml; valeric acid and 0.55 ml; *iso*-valeric acid per litre of 0.2M NaOH.
[d] Composition : 20.5 g: sodium sulphite ($Na_2S.9H_2O$) in 1l distilled water with nitrogen gas bubbling through it.
[e] Composition : 0.0204 g; biotin, 0.0205 g; folic acid, 0.1740 g; calcium D- pantothenate, 0.1640 g; nicotinamide, 0.1640 g; riboflavin, 0.1640 g; thiamin HCl, 0.1640 g; pyridoxine HCl, 0.0204 g; *para*-amino benzoic acid, 0.0205 g; cyanocobalamin (vitamin B12), in 1l of solution containing 54.7g KH_2PO4.
[f] Composition : 82 g; Na_2CO_3 (sodium carbonate anhydrous) per boiled distilled water with CO_2 bubbling through it.

Table 2: Total starch (TS), resistant starch (RS), fermentative end-products and short-chain fatty acid ratios for selected starches incubated in slurries of mixed caecal bacteria

Starch source	Total starch (g/100g)	RS as % of TS (g/100g TS)	Total Short Chain Fatty Acid (TSCFA)	Acetic acid	Propionic acid	Butyric acid
Sago	84.81[a]	2.17[de]	24.29[de]	14.40[de]	2.44[d]	7.45[de]
Sweet potato	83.79[a]	3.15[cde]	31.92[c]	18.62[c]	3.33[bc]	9.97[bc]
Potato starch	77.39[cd]	24.08[a]	16.05[f]	10.66[e]	1.52[e]	3.87[f]
Arrowroot	78.35[bc]	17.19[b]	24.28[de]	13.78[de]	2.66[cd]	7.85[cde]
Rice	73.52[d]	3.65[cde]	57.78[a]	32.96[a]	5.54[a]	19.28[a]
Wheat	83.73[a]	0.78[e]	38.73[b]	23.31[b]	3.62[b]	11.80[b]
Tapioca	77.25[cd]	5.49[c]	22.56[e]	13.62[de]	2.42[d]	6.52[e]
Corn	81.98[ab]	1.59[de]	27.22[de]	16.02[cd]	2.85[cd]	8.35[cde]
Cassava pulp	68.83[e]	3.31[cde]	30.03[cd]	17.41[cd]	3.17[bc]	9.45[cd]
Sweet potato root meal	57.51[f]	4.24[cd]	29.60[cd]	16.62 [cd]	3.17[bc]	9.81[bc]
SEM	1.53	1.03	2.14	1.28	0.21	0.71
Probability (*P*)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] Values are Mean ±Standard deviation [b] Total short-chain fatty acid = acetic acid + propionic acid + butyric acid (BEDNAR *et al*., 2001)

[c] Values not sharing the same superscripts along the same column are different

SCFA: Short-chain fatty acid; AAE: Acetic acid equivalents

Table 3: Short-chain fatty acid ratios and fermentation ratios for selected starches incubated in slurries of mixed caecal bacteria

Starch source	Short chain fatty acid (SCFA) ratios			Fermentation ratios		
	Acetic acid/TSCFA	Propionic acid/TSCFA	Butyric acid/TSCFA	Acetic acid/RS	Propionic acid/RS	Butyric acid/RS
Sago	0.59[bc]	0.10[ab]	0.31[ab]	0.52[a]	0.09[a]	0.27[a]
Sweet potato	0.58[bcd]	0.10[ab]	0.31[ab]	0.20[c]	0.04[c]	0.11[c]
Potato	0.66[a]	0.09[cd]	0.24[c]	0.12[d]	0.02[e]	0.04[e]
Arrowroot	0.57[cd]	0.11[a]	0.32[a]	0.15[cd]	0.03[cd]	0.08[cd]
Rice	0.57[cd]	0.09[cd]	0.33[a]	0.50[a]	0.08[a]	0.29[a]
Wheat	0.60[b]	0.09[cd]	0.30[ab]	0.31[b]	0.05[b]	0.16[b]
Tapioca	0.61[b]	0.11[a]	0.29[b]	0.15[cd]	0.03[d]	0.07[de]
Corn	0.59[bc]	0.10[ab]	0.31[ab]	0.19[c]	0.03[cd]	0.10[cd]
Cassava pulp	0.58[bcd]	0.11[a]	0.31[ab]	0.19[c]	0.03[cd]	0.10[cd]
Sweet potato root meal	0.56[d]	0.11[a]	0.33[a]	0.19[c]	0.04[c]	0.11[c]
SEM	0.02	0.00	0.01	1.17	0.20	0.62
Probability (*P*)	<0.0001	<0.0001	<0.0001	<0.0001	<0.00010	<0.0001

[a] Values are Mean ± Standard deviation [b] Values not sharing the same superscripts along the same column are different [c] TSCFA: Total short-chain fatty acid, [d] RS: Resistant starch

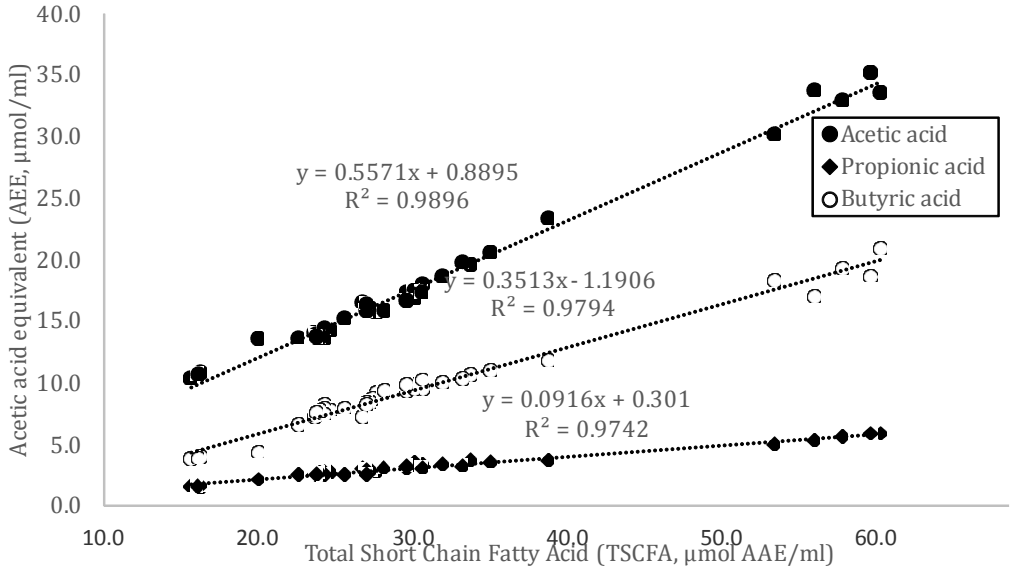

Figure 1: Graph of linear regression models for proportion of acetic acid, propionic acid and butyric acid versus total short chain fatty acid produced during fermentation of selected starches in slurries of mixed caecal bacteria

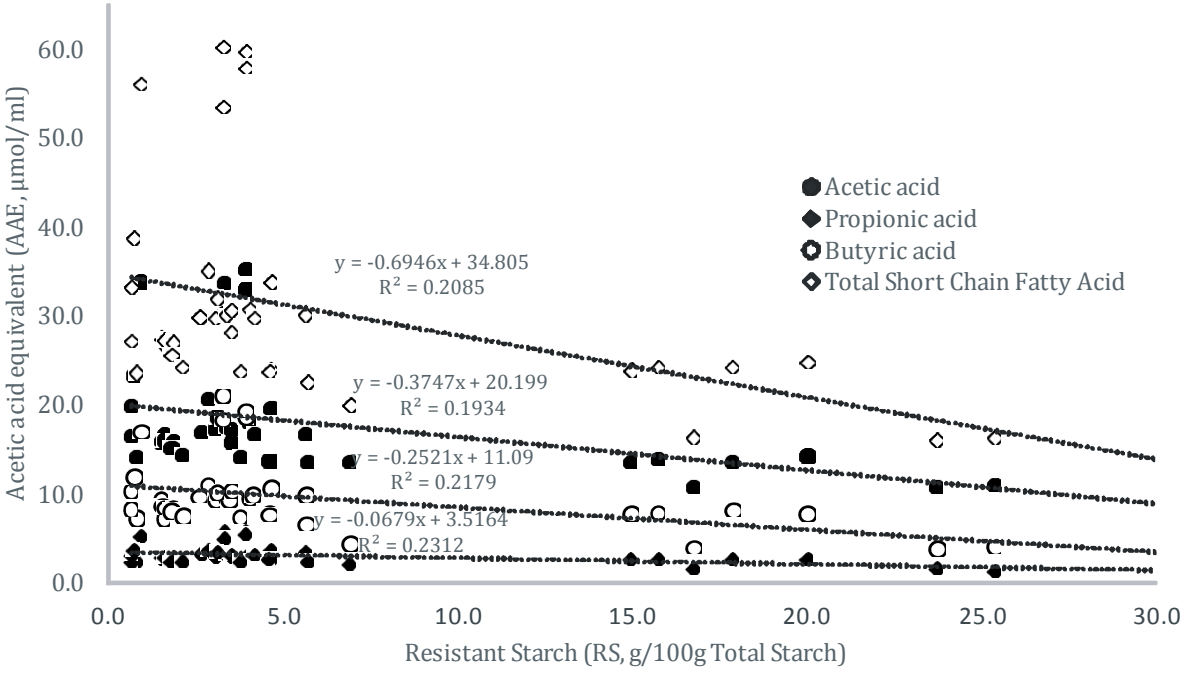

Figure 2: Graph of linear regression models for proportion of acetic acid, propionic acid, butyric acid and total short chain fatty acid versus resistant starch (RS) in selected starches fermented in slurries of mixed caecal bacteria

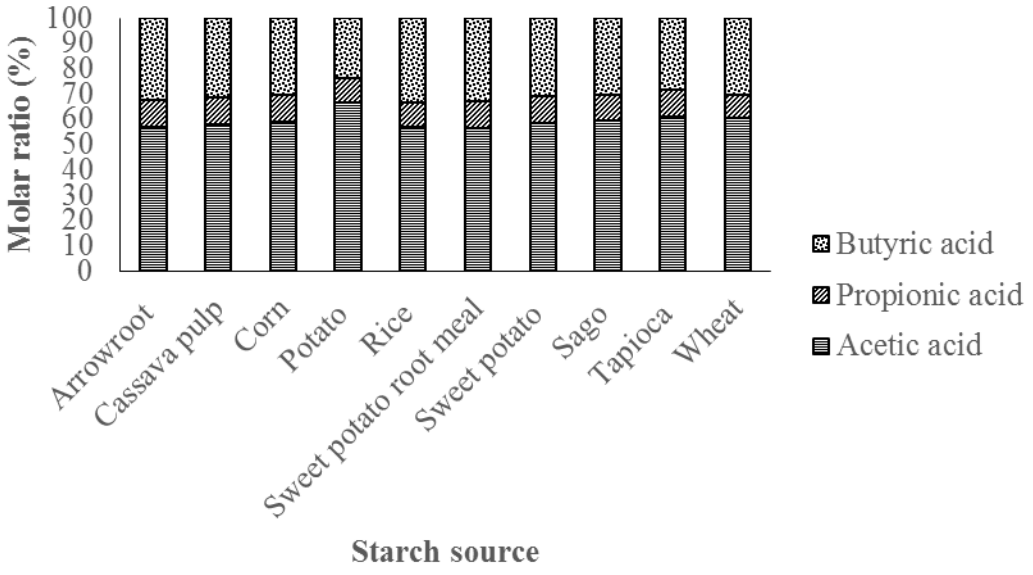

Figure 3: Molar ratios of acetic, propionic and butyric acids produced by fermentation of selected starches in slurries of mixed caecal bacteria *in vitro*