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Biotechniques to assess the fermentation of resistant starch in the mammalian gastrointestinal tract

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Abstract

Energy salvage from the fermentation of retrograded resistant starch (Novelose™) was investigated using the rat as a model for man. Total bacterial fermentation of resistant starch specifically in the large gut regions (caecum, proximal colon and distal colon) was determined with reference to a dietary marker (TiO_2). Total short-chain fatty acid and lactate production by fermentation in vivo was estimated using stoichiometrics (mol mol^{-1} glucose in resistant starch) obtained from resistant starch fermentation of recovered gut contents in vitro. Utilisation of short-chain fatty acids and lactate represented the difference between total production less equilibrium values of these components in the gut contents as isolated. 55–62% of the gross energy of the resistant starch fermented was salvaged by the host.

Keywords: Fermentation; Mammalian gastrointestinal tract; Resistant starch

1. Introduction

For nutritional purposes, starch may be classified into three types, depending on the extent to which it is digested in the mammalian gastrointestinal tract (Englyst et al., 1992). Rapidly digestible starch (RDS), present in freshly cooked, starchy foods, is rapidly and completely digested in the small intestine. Slowly digestible starch (SDS), as in most raw cereals, is completely digested in the small intestine but at a slower rate. Resistant starch (RS) is resistant to normal digestion in the small intestine. RS is subdivided into 3 fractions: RS1 is physically inaccessible starch found in partially milled grains and seeds; RS2 are resistant granules, such as those in raw potato and banana; and RS3 is retrograded starch, formed in processed foods on cooling (cooled, cooked potato, bread and cornflakes). Since most starchy foods in the human diet are either cooked or processed prior to consumption, varying amounts of RS3 will be produced that resists normal digestion in the small intestine of man and laboratory models.

The formation of RS3 is related to the amylose content of the initial starch (Pomeranz, 1992) and to the number of processing cycles (heating and cooling) that it is subjected to (Berry, 1986). Heat/moisture treatment results in the disruption of the native granular structure and a partial solubilisation of the starch polysaccharides. Granular structures which have been gelatinised by heating in water are readily hydrolysed by amylolytic enzymes, but on cooling to room temperature the solubilised polysaccharides can retrograde (Miles et al., 1985). Formation of the retrograded starch fraction is the result of reassociation of predominantly amylose chains into 10 nm double helical regions, with an approximate degree of polymerisation of 43, packed in a B-crystalline structure (Jane and Robyt, 1984; Sievert et al., 1991). The helical structures are highly resistant to hydrolysis by α-amylases, but are interspersed with more susceptible amorphous regions.

RS3, along with other forms of resistant starch, consumed in the diet escapes digestion in the small intestine and enters the large bowel. Here, it provides a substrate for fermentation by the diverse population of anaerobic bacteria that inhabit the lumen (Cummings and Englyst, 1991). The production of bacterial mass is promoted together with short-chain fatty acids (SCFA), principally acetate, propionate and butyrate, lactate and gases (CO_2, CH_4 and H_2).

Some of the SCFA (acetate and propionate) and lactate will be absorbed through the intestinal wall, transported to the liver via the portal circulation and subsequently utilised by the host. Butyrate will be used specifically by the colonocytes. Bacterial fermentation, therefore, represents a

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Table 1
Composition of fully synthetic trial diets fed to rats

Component	g kg^{-1} fresh weight
Casein	149
L-methionine	1
Vegetable oil	50
Mineral mix[a]	50
Vitamin mix[b]	10
Starch[c]	740

[a]KH_2PO_4 434.0 g kg^{-1}, $MgSO_4.7H_2O$ 47.6 g kg^{-1}, $CaCO_3$ 350.0 g kg^{-1}, NaCl 138.5 g kg^{-1}, $FeSO_4.7H_2O$ 21.6 g kg^{-1}, $MnSO_4.5H_2O$ 4.6 g kg^{-1}, $ZnSO_4.7H_2O$ 1.8 g kg^{-1}, $CuSO_4.5H_2O$ 0.9 g kg^{-1}, KI 0.8 g kg^{-1}, $CoCl_2$ 0.2 g kg^{-1}.
[b]Vitamin diet fortification mixture (ICN Biomedicals Inc, Ohio. Cat. 904654).
[c]Starch fraction was a mixture of Novelose and a fully digestible maize starch, proportioned according to the level of RS3 required (see Table 2).

salvage of energy from an otherwise indigestible fraction of the food.

The studies reported here were designed to quantify the utilisation of RS3 via fermentation as directly as possible and determine the contribution that this salvage makes to the overall energy economy of the model.

2. Materials and methods

2.1. Materials

Novelose™ was supplied by National Starch (Manchester, U.K.). Maize starch for inclusion in rat diets was obtained from Cerestar U.K. Ltd (Manchester).

Termamyl (Type L 120 KNU/g) and amyloglucosidase (300 AG/ml) enzymes were from Novo Nordisk Bioindustries Ltd (Solihull).

Test kits for glucose and L/D-lactic acid were obtained from Boehringer (Mannheim, Germany).

2.2. In vivo studies

Experimental animals were specific pathogen-free male Wistar weanling rats (B and K Universal, Glasgow). Animals were housed individually in wire mesh cages with wire bottoms. Trial room environment was maintained at 21°C, with 56% humidity and a light:dark cycle of 12:12 h. Body weight changes were recorded every 2 days. Ad libitum access was permitted to water and feed.

Table 2
Composition of starch fraction of trial diets fed to rats, formulated to contain various levels of RS3

Group no.	Novelose (g kg^{-1})	Maize (g kg^{-1})	RS_3 (g kg^{-1})
1	0	740	0.0
2	50	690	16.7
3	100	640	33.4
4	200	540	66.8
5	300	440	100.2
6	400	340	133.6
7	600	140	200.4

42 animals, mean start weight 90.1 ± 6.3 g, were randomly assigned to dietary groups 1–7 (see Table 1 and Table 2), each group being fed a different incorporation level of Novelose in their ration. All the diets contained 0.5% w/w TiO_2. Diets were offered mixed 1:1 with a ground commercial chow for 2 days, then fed undiluted for an experimental period of 8 days. Faecal collections were made from days 6 to 8. At sacrifice, material was taken from the ileum, caecum, proximal and distal colon and terminal faecal pellets collected. Approximately 0.1 g of fresh material was taken from each region of the gut and placed immediately into liquid N_2 for SCFA and lactic acid analysis (termed 'equilibrium' values).

10 animals, mean starting weight 94.3 ± 7.1 g, were fed a diet containing 50% Novelose and 24% maize starch for an identical experimental period. At the end of the feeding period, material was collected from the caecum, proximal and distal colon. Contents were pooled and mixed under N_2 for use in in vitro fermentation studies.

2.3. Analytical methods

2.3.1. Total starch estimation

Starch was estimated using the method of Nicol (1998) developed in this laboratory. Samples ($n > 2$) containing 40–50 mg starch were mixed with 0.5 ml distilled water in 40 ml screw top digestion tubes for 15 min at room temperature. Duplicate samples of 40–50 mg soluble wheat starch were included as standards. Several tubes, containing enzymes and buffer only, were treated similarly throughout to act as controls (blanks). Samples were solubilised by the addition of 2 ml 2 M KOH and stirred constantly for 1 h at room temperature. Following this, the pH was adjusted to 6.5 by the addition of 4 M HCl (the volume required was first tested on blank tubes, which were then discarded) and 2 ml 0.1 M MES buffer [2(N-morpholino ethane sulphonic acid) containing 1 mM $CaCl_2$, pH 6.5]. 10 ml of termamyl were added and the tubes incubated in a water bath at 100°C for 1.5 h. A second 10 ml of termamyl was added and the tubes incubated at 90°C for a further 1.5 h. The pH was adjusted to 4.6 with 0.1 M sodium acetate buffer (pH 4.1, pre-heated to 58°C), 0.1ml amyloglucosidase added and the sample incubated at 58°C for 2 h. Following overnight storage at 4°C, digests, standards and controls were diluted into 200 ml with distilled water and mixed thoroughly. Aliquots were centrifuged at 2000 *g* for 5 min and the glucose content of the supernatant was determined using a glucose oxidase method (Boehringer Test Kit no. 124 036).

2.3.2. SCFA and lactate determination

Duplicate intestinal content samples of approximately 0.1 g fresh weight were extracted with 2 ml absolute ethanol in 12 ml centrifuge tubes and 0.2 ml of the internal standard

1 mg ml^{-1} 3-methyl-valeric acid were added. Tubes were mixed briefly several times over a period of 5 min and centrifuged at 3800 *g* for 5 min. 1 ml of the supernatant was transferred to a 50 ml round-bottomed flask with 3 drops of 1 M NaOH. Samples were then evaporated to dryness at less than 25°C. Immediately prior to analysis, 15 μl distilled water and 5 μl ortho-phosphoric acid were added. 0.5 μl aliquots were analysed by isothermal (100°C) GC (Pye104), on dual packed columns (6 ft × 2 mm) Supelco GP 10% SP1200/1% H_2PO_4 on Chromasorb WAW using argon (20 ml min^{-1}) as carrier gas. Detection was by FID. Peak integration was performed using a computing integrator (Pye Unicam CDP1).

The amounts of the SCFA acetate, propionate, iso-butyrate, *n*-butyrate, *iso*-valerate and *n*-valerate were calculated with reference to the internal standard, 3-methyl-valerate.

L/D-lactic acid was determined enzymatically on the remaining alcoholic extract using standard test LDH (Boehringer Test Kit no. 1112 821).

2.3.3. TiO_2 analysis

TiO_2 in faeces and intestinal contents was determined colorimetrically by the method of Short et al. (1996). Approximately 50 mg aliquots of freeze dried sample ($n = 2$) were ashed in 40 ml porcelain crucibles at 580°C in a muffle furnace (S1204 PID, Stuart Scientific, Redhill, Surrey) for a minimum of 4 h. After cooling, the samples were heated with 10 ml 40% H_2SO_4 until the ash dissolved and the solution became translucent. Following dilution with 10 ml distilled water, samples were filtered through a Whatman 541 filter paper into 50 ml volumetric flasks containing 10 ml 30% H_2O_2. The volume was made up to 50 ml with distilled water and the absorbance of the resulting solution read at 410 nm. TiO_2 concentration was ascertained by reference to a standard curve of 0–50 mgTiO_2 ml^{-1}.

2.3.4. In vitro fermentation of intestinal contents

A minimum of 0.1 g of fresh intestinal contents were weighed directly into sets of 4 ml screw-top glass vials fitted with aluminum lined caps (Supelco Inc, Poole, Dorset, U.K.). Vials were gassed with N_2 to maintain anaerobiosis and incubated at 37°C. Fermentation was terminated at time = 0 min, and approximately 30 min intervals up to a maximum of 3 h, by the addition of 2 ml absolute ethanol and 0.2 ml 1 mg ml^{-1} 3-methyl-valeric acid. Samples were transferred to centrifuge tubes and centrifuged at 3800 *g* for 5 min. The supernatant was assayed for SCFA and L/D-lactic acid as detailed. Any remaining ethanol was removed from the surface of the pellet by aspiration and the samples subsequently frozen to −20°C and freeze dried. The total starch content of the material was determined.

2.4. Estimation of contribution of RS3 fermentation to energy economy

To obtain accurate estimates of the contribution of RS3 fermentation to the energy economy of the rat, quantitative data on the fermentation products (SCFA and lactate) absorbed in the different regions of the gut were required. Such data was calculated from the following experimentally measured parameters:

1. RS3 escaping digestion in the ileum, i.e. the amount of RS3 entering the large bowel.
2. Quantitative estimates of RS3 fermentation in each section of the large gut (caecum, proximal and distal colon).
3. Quantitative values for the products of the above fermentation that are utilised by the rat as an energy source.
4. Proportions of the above products that are absorbed and utilised by the rat.

1 and 2 were estimated by the use of a suitable dietary marker (TiO_2). RS3 and TiO_2 were determined on ileal, caecal, proximal colon (PC), distal colon (DC) and faecal material recovered at the end of the experimental period. Thus, it follows that:

$$\text{a. RS3 escaping ileal digestion} = \frac{\text{Ileal RS3}}{\text{Ileal TiO}_2} \times \text{TiO}_2 \text{ intake (mg d}^{-1}\text{)}$$

$$\text{b. RS3 fermented in caecum} = \frac{\text{Caecal RS3}}{\text{Caecal TiO}_2} \times \text{TiO}_2 \text{ intake (mg d}^{-1}\text{)}$$

c.

$$\text{RS3 fermented in PC} = a - b - \left[\frac{\text{PC RS3}}{\text{PC TiO}_2} \times \text{TiO}_2 \text{ intake (mg d}^{-1}\text{)}\right]$$

d.

$$\text{RS3 fermented in DC} = a - (b + c) - \left[\frac{\text{DC RS3}}{\text{DC TiO}_2} \times \text{TiO}_2 \text{ intake (mg d}^{-1}\text{)}\right]$$

e.

$$\text{RS3 fermented in the faeces} = a - (b + c + d) - \left[\frac{\text{Faecal RS3}}{\text{Faecal TiO}_2} \times \text{TiO}_2 \text{ intake (mg d}^{-1}\text{)}\right]$$

3. The assumption was made that the fermentative production of SCFA and lactate from RS3 in the digesta in specific regions of the gut was the same as that in vitro using material recovered at the termination of the balance trial.

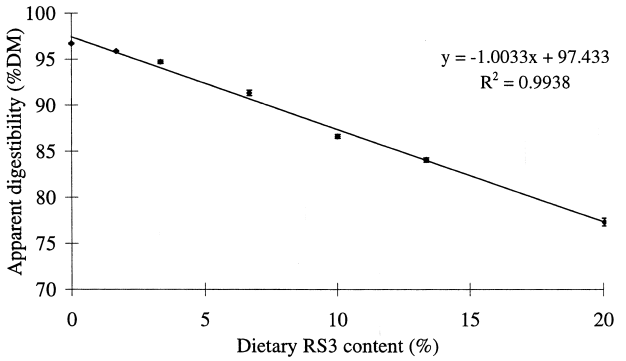

Fig. 1. The relationship between apparent dry matter (DM) digestibility and RS3 incorporation level of synthetic diets supplied to rats. The values were calculated using DM:TiO_2 ratios in feed and faeces collected on days 6–8 of the trial period.

Thus, SCFA (acetate, propionate and butyrate) and D- and L- lactate production (mmol) per RS3 glucose unit fermented could be defined quantitatively as:

Total SCFA/lactate produced = [Total RS3 glucose units fermented (mmol)] x [SCFA/lactate production mmol−1].

4. For caecum, PC and DC respectively, the utilisation of fermentation products was calculated as follows:[Total SCFA/lactate production (mmol d^{-1}] - {[Equilibrium values for SCFA/lactate in recovered digesta × TiO_2 intake (mg d^{-1})]/[TiO_2 in recovered digesta (mg)]}.

The sum of caecal, PC and DC represents the total utilisation of RS3 via fermentation. It was assumed that any production of SCFA and lactate in the faeces would not be recovered by the animal.

3. Results

The apparent DM digestibility was inversely linearly related to dietary RS3 inclusion level ($r^2 = 0.99$) (Fig. 1).

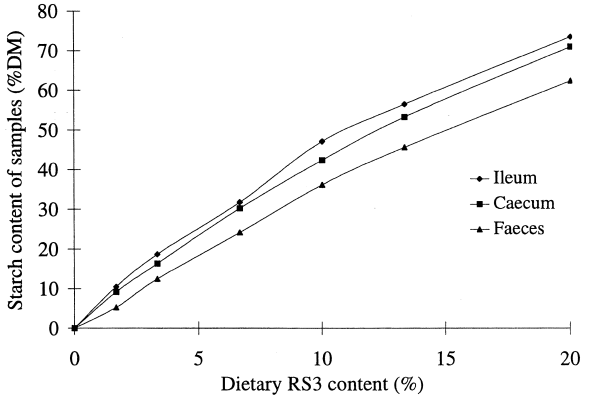

Fig. 2. The relationship between the RS3 content of DM of gut isolates and dietary incorporation level of RS3 supplied to weanling rats. The starch measured was total starch, which has previously been shown to be all RS in regions below the mid-ileum (unpublished data). The ileal region in this case was the terminal 6 cm of the small intestine.

Levels of RS3 in the DM isolated from all regions of the gut increased proportionately with increasing dietary inclusion level (Fig. 2).

The amount of RS3 via fermented in each region of the gut was increased with dietary RS3 content but the increment varied with gut region. Overall, a doubling of dietary RS3 resulted in a proportional increase in fermentation (Table 3). Estimates of the total production of SCFA and lactate from RS3 fermentation were obtained by means of an in vitro fermentation system (Fig. 3). The qualitative and quantitative pattern of SCFA production (mols of SCFA and lactate per mol of glucose fermented) was found to be similar in the caecum, proximal and distal regions of the colon.

The total production of SCFA and lactate at each RS3 dietary inclusion level was calculated as the product of total starch glucose fermented and the stoichiometric values per mol of starch glucose fermented in vitro (Table 4). The net absorption of SCFA and lactate from the gut represented the difference between the total production and equilibrium values of these components in the gut contents as isolated. The proportion of SCFA and lactate, from RS3 fermentation, absorbed by the model represented a salvage of 55–62% of the gross energy of the starch utilised in the lower gut (Table 5).

4. Conclusion

In vitro systems have been used as an alternative to invasive techniques to quantify the products of RS fermentation. Many studies have involved the use of human faecal inocula to ferment indigestible polysaccharides in vitro as a method of quantifying the production of SCFA and lactate in vivo (Tomlin et al., 1986; Englyst and Macfarlane, 1986; Silvester et al., 1995). Although these methods have provided information on the products of fermentation of RS, they are considered to be inaccurate for determining the stoichiometry of RS utilisation and the exact quantities of SCFA and lactate produced as a result of fermentative processes in vivo. The population of bacterial flora found in the faeces is only representative of the luminal flora of the recto-sigmoid region, which differs markedly from the population of flora found closely associated with the mucosal surface and villous crypts of the colon (Hill and Draser, 1975). Population differences were further substantiated by Monsma and Marlett (1996) who reported that the efficiency of bacterial utilisation of carbohydrate from pea fibre was greater in the ileal excreta of rats incubated with caecal inocula as compared to faecal inocula. Edwards et al. (1996) incubated faecal slurries from healthy human subjects with retrograded amylose. In addition to the RS3 fraction, the preparation also contained appreciable amounts of readily digestible starch that would be completely hydrolysed in the ileum and under normal circumstances would never reach the colon. Microbial fermentation of physiologically available starch in addition to RS3 would

Table 3
Total glucose utilised via fermentation (mmol) in different regions of the gut at various dietary RS3 inclusion levels

RS3 (%)	Caecum	Proximal colon	Distal colon	Faeces	Total utilised (mmol glucose)
0	0.00	0.00	0.00	0.00	0.00
10	1.35	0.60	0.62	0.24	2.81
20	3.03	1.16	0.52	0.96	5.66
Pooled SE	0.26	0.19	0.20	0.15	0.32

Glucose utilisation at each stage of the gut was calculated from starch:TiO_2 ratios in the DM. TiO_2 was used to calculate the DM flow through each region of the gut and, hence, the disappearance of starch from the system determined.

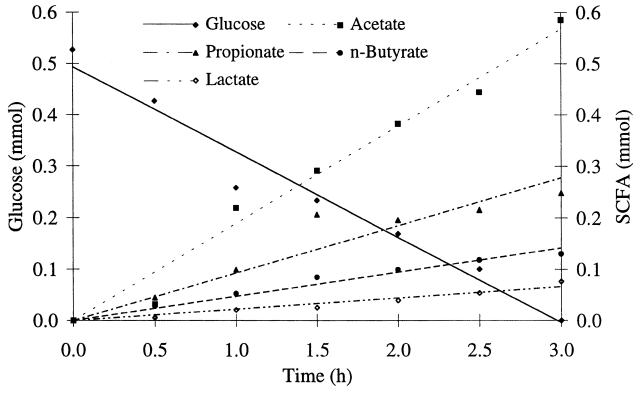

Fig. 3. The production of SCFA and lactate by bacterial fermentation of RS3 in caecal contents in vitro at 37°C, under N_2. (r^2; glucose = 0.98, acetate = 0.97, propionate = 0.91, *n*-butyrate = 0.98, lactate = 0.98). The pattern of SCFA production was similar in both proximal and distal colon.

probably have altered both the quantities and types of SCFA produced. Additionally, the use of different media and different innocula/substrate concentrations in the current in vitro investigations have been shown to affect net SCFA production during the in vitro fermentation of ordinary maize starch with rat caecal inocula (Stevenson et al., 1997).

In the investigations reported here, the combination of direct digestibility measurements, with respect to TiO_2, and in vitro fermentation studies enabled an accurate estimation of the utilisation of the products of RS3 fermentation to be made. The approach employed in these studies was considered to give a better representation of the fermentative processes that were occurring in the large intestine, as:

1. Material was taken directly from the main fermentative sites in the large intestine, as opposed to using faecal inocula.
2. Only physiologically resistant starch was present in the incubation mixture as any digestible starch was removed by enzymatic degradation in the ileum.
3. The system utilised the natural buffering capacity of the intestinal digesta, as pH changes over the incubation period were insufficient to affect the rates of fermentation (unpublished data).

Bacterial fermentation of RS3 facilitated a considerable salvage of energy by the model. Energy not absorbed by the animal as SCFA or lactate would be utilised directly by the bacteria or lost as non-utilisable bacterial products (CO_2, CH_4 and H_2).

Table 4
In vitro stoichiometry of SCFA and lactate production from RS3 fermentation

	Production (mmol) per mmol RS3 glucose fermented			
	Acetate	Propionate	*n*-Butyrate	Lactate
Mean	0.94	0.31	0.19	0.18
SE	0.12	0.02	0.08	0.03

Table 5
Production and absorption efficiency of RS3 fermentation products and proportion of Gross Energy (GE) salvaged by fermentation at various dietary inclusion levels

RS3 (%)	Glucose fermented (mmol)	Total SCFA/lactate production (mmol)	Net SCFA/lactate absorption (mmol)	Proportion SCFA/lactate absorbed (%)	Proportion of GE of glucose salvaged (%)
0	0.00	0.00	0.00	0.0	0.0
10	2.81	4.30	3.89	90.0	61.0
20	5.66	8.66	8.14	94.0	59.0
Pooled SE	0.32	0.33	0.13	31.5	18.5

Net SCFA and lactate absorption and utilisation was represented as the difference between total production less equilibrium values of these products in the recovered digesta. The Gross Energy (GE) salvage is expressed as a proportion of total RS3 glucose entering the lower gut.

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