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Influence of a diet rich in resistant starch on the degradation of non-starch polysaccharides in the large intestine of pigs

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ABSTRACT

To investigate the effect of resistant starch to the degradation of other non-starch polysaccharides (NSPs) in the large intestine of pigs, two groups of pigs were fed either a diet containing digestible starch (DS) or a diet containing resistant starch (RS). Both diets contained NSPs from wheat and barley. Digesta from different parts of the large intestine were collected and analysed for sugar composition and carbohydrate-degrading-enzyme activities. Resistant starch, as well as β-glucans and soluble arabinoxylan, was utilised mainly in the caecum. The utilisation of β-glucans and soluble arabinoxylan in the caecum was higher in DS-fed pigs than in RS-fed pigs. Analyses on carbohydrate-degrading-enzyme activities demonstrated that microbial enzyme production was stimulated according to the diet composition, and the enzyme profile throughout the large intestine of RS-fed pigs indicated that the presence of resistant starch shifted the utilisation of NSPs to more distal parts of the colon.

1. Introduction

Dietary fibres include carbohydrates with diverse physico-chemical properties. The effects of one fibre, therefore, may not be the same as the effects of another fibre (Cummings, Edmond, & Magee, 2004). Resistant starch is a dietary fibre that has various health benefits, including improving bowel health, acting as a prebiotic, and increasing satiety (Nugent, 2005).

The benefits of resistant starch are in general assumed to be caused mainly by its fermentation in the large intestine and the short chain fatty acids produced during the fermentation (Topping & Clifton, 2001) although the effects of resistant starch may depend on the types (Dongowski, Jacobasch, & Schmiedl, 2005; Lesmes, Beards, Gibson, Tuohy, & Shimoni, 2008; Martínez, Kim, Duffy, Schlegel, & Walter, 2010). In order to understand the effects of resistant starch on various health aspects in the large intestine, animal models are often employed. Pigs are regarded as the most suitable model for human in relation to gastrointestinal system (Bergman, 1990). In pigs, resistant starch has been shown to be able to increase the production of short chain fatty acids, especially butyrate, and bacterial diversity in the large intestine (Martin, Dumon, & Champ, 1998; Martínez-Puig, Castillo, Nofrarias, Creus, & Pérez, 2007), and to influence nutrient utilisation (Schrijver, Vanhoof, & Vande Ginste, 1999; Rideout, Liu, Wood, & Fan, 2008).

The intake of resistant starch is usually accompanied by the consumption of other dietary fibres, which are grouped as non-starch polysaccharides (NSPs). Despite the many studies about the effects of resistant starch to various aspects in the large intestine, the effect of resistant starch to the degradation of specific NSPs by the large intestinal microbiota has seldom been mentioned. The large intestinal microbiota produces enzymes to degrade polysaccharides (Louis, Scott, Duncan, & Flint, 2007). Resistant starch may modify the microbial composition in the large intestine. For example, resistant starch stimulated the growth of *Bifidobacterium* spp. in pigs (Regmi, Metzler-Zebeli, Gänzle, van Kempen, & Zijlstra, 2011) and increased the population of *Ruminococcus bromii* in humans (Abell, Cooke, Bennett, Conlon, & McOrist, 2008; Walker et al., 2011). Hence, it may also change the degradation of NSPs in the large intestine. In order to estimate the potential digestibility of various substrates by microbiota, an approach employing measurements of enzyme activities towards different polysaccharides has been developed (Michalet-Doreau, Fernandez, & Fonty, 2002; Williams & Withers, 1991).

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In the present study, an *in vivo* experiment was performed, using pigs as models for humans, to assess the effect of resistant starch on the production and composition of short chain fatty acids, genetic expressions in the large intestinal tissue, and interaction between resistant starch and NSPs. This paper is focused on the interaction between resistant starch and NSPs, by measuring the sugar composition and enzyme activities in the large intestinal digesta.

2. Materials and methods

2.1. Experimental

The experimental procedures were approved by and conformed to the requirements of the Animal Care and Use Committee of Wageningen University, Wageningen, The Netherlands.

2.1.1. Animals and pens

Twenty female pigs (22-month-old, Landrace, PIC Benelux B.V., Rosmalen, The Netherlands) with body weight 272.5 ± 3.9 kg were housed in pens of 11 m^2, each containing two pigs. Each pen was equipped with two drinking nipples and two feeding troughs. Artificial lights were on from 06:30 h until 22:00 h and dimmed during the night.

2.1.2. Experimental diets

The compositions of the experimental diets are presented in Table 1. The main source of starch in the digestible starch (DS) diet was pregelatinised potato starch (Paselli WA4, AVEBE, Foxhol, The Netherlands). In the resistant starch (RS) diet, the pregelatinised starch was replaced by retrograded tapioca starch (Actistar, Cargill, Amsterdam, The Netherlands) based on dry matter basis. According to the supplier, the starch was produced by enzymatic de-branching of tapioca starch, followed by retrogradation, resulting in RS type 3 which was at least 50% resistant starch based on an assay using resistant starch kit (Megazyme, Bray, Ireland).

Each experimental diet was given to 10 pigs. Siblings were equally distributed between the two diet groups. The pigs were fed about 1.13 times of their energy requirements for maintenance with half of the daily amount in the morning (07:00 h) and the other half in the afternoon (17:00 h). The pigs were fed the experimental diet for 2 weeks. In the third week, digesta samples were collected from the pigs.

Table 1
Composition of the experimental diets (g/100 g).

Ingredients	Digestible starch diet[a]	Resistant starch diet
Pregelatinised potato starch	35.00	
Retrograded tapioca starch		34.26
Wheat	24.90	25.18
Barley	15.00	15.17
Maize gluten flour	10.00	10.11
Potato protein	5.00	5.06
$CaCO_3$	1.70	1.72
Soy oil	1.50	1.52
Animal fat	1.50	1.52
$NaHCO_3$	1.50	1.52
Mono calcium phosphate	1.10	1.11
Pig premix for pigs > 40 kg[b]	1.00	1.01
KCl	1.00	1.01
NaCl	0.50	0.51
L-Lysine HCl	0.15	0.15
Flavours	0.15	0.15

[a] The amount of digestible starch diet given to the pigs was 1.12% higher than the amount of resistant starch diet, due to different moisture contents between the diets.

[b] The premix provided the following per kg food (of the control diet): vitamin A: 10,000 IU; vitamin D3: 2000 IU; vitamin E: 25 mg; vitamin K3: 1.0 mg; vitamin B1: 0.75 mg; vitamin B2: 4.0 mg; vitamin B6: 1.0 mg; vitamin B12: 15 μg; niacin: 20 mg; D-panthothenic acid: 13 mg; choline chloride: 300 mg; folic acid: 2.5 mg; biotin: 0.1 mg; Fe: 80 mg ($FeSO_4 \cdot H_2O$); Cu: 10 mg ($CuSO_4 \cdot 5H_2O$); Mn: 30 mg (MnO); Zn: 60 mg ($ZnSO_4 \cdot H_2O$); Co: 0.20 mg ($CoSO_4 \cdot 7H_2O$); I: 0.75 mg (KI); Se: 0.20 mg (Na_2SeO_3).

2.1.3. Digesta collection from the large intestine

The pigs were fed about 5 h prior section to ensure presence of fresh digesta. Stunning was done by head-only electrocution (electrodes placed below the base of the ears on either side of the head) followed by exsanguination within 15 s of initial stunning of the animal. Exsanguination was done by severance of the major blood vessels in the neck.

Immediately after exsanguination, the abdominal cavity was opened and the gastrointestinal tract from stomach to anus was removed from the cavity. The large intestine was separated from the other parts of gastrointestinal tract, and was divided into caecum (Cae), proximal colon (pCol), proximal mid-colon (pmCol), distal mid-colon (dmCol), and distal colon (dCol). The digesta in every section of the large intestine was collected and immediately stored frozen (−20 °C). The samples were freeze-dried before further analyses. Samples from four pigs per experimental diet were analysed. The samples were taken from pairs of sisters, to minimise variation that might occur due to genetic background.

2.2. Sample fractionation

The freeze-dried digesta was separated into soluble and insoluble fractions by suspending 1.25 g of freeze-dried digesta in 150 mL water. The suspension was stirred for 20 min and subsequently centrifuged (10,000 × *g*; 20 min). The supernatant was collected, and the pellet was suspended in 100 mL water and then stirred for 20 min. The suspension was centrifuged (10,000 × *g*; 20 min) and the supernatant was combined with the first one. The whole procedure was performed at 4 °C. The combined supernatant was then boiled for 10 min to inactivate enzymes and freeze-dried, prior to further analyses.

2.3. Chemical and enzymatic analyses

2.3.1. Extraction of non-starch polysaccharides

Samples were defatted using acetone. After centrifugation and removal of the supernatant, the samples were pre-dried at 75 °C, followed by drying at 50 °C overnight. The defatted samples were then milled using a ball milling apparatus (MM2000, Retsch, Haan, Germany).

Starch was gelatinised by mixing 300 ± 5 mg of defatted sample with 2 mL DMSO followed by boiling for 30 min. Next, the starch was enzymatically degraded by adding 7.5 mL sodium phosphate buffer (0.08 M, pH 6.0) and 50 μL thermostable α-amylase (EC 3.2.1.1, Megazyme, Bray, Ireland) followed by incubation in boiling water bath for 30 min. After incubation, the pH was adjusted to 4.0–4.6 using 0.325 N HCl. Subsequently, 50 μL amyloglucosidase (Sigma–Aldrich, Schnelldorf, Germany) was added. The samples were subsequently incubated at 60 °C for 1.5 h. Following incubation, enzymes were inactivated by heating in a boiling water bath for 10 min.

Precipitation and washing of the polysaccharides for total and insoluble NSPs were performed as described elsewhere (Englyst, Quigley, & Hudson, 1994), except that the overnight drying was performed at 50 °C instead of 80 °C. The dried sample was further analysed for sugar content and composition. Soluble NSP content and composition were calculated from the difference between total and insoluble NSP.

2.3.2. Sugar composition

Prehydrolysis was performed in 72% (w/w) sulphuric acid at 30 °C for 1 h followed by hydrolysis in 1 M sulphuric acid at 100 °C

for 3 h. After derivatisation into alditol acetates, monosaccharides were analysed using gas chromatography (Englyst & Cummings, 1984). Inositol was used as internal standard.

2.3.3. Starch and mixed-linkage β-glucan content

Starch content was analysed using total starch kit from Megazyme (Bray, Ireland). Glucose and maltodextrins were regarded as starch degradation products, and were included in the analysis. Mixed-linkage β-glucan content was analysed using mixed-linkage β-glucan kit (Megazyme).

2.3.4. Protein extraction

The proteins, including enzymes, in the digesta were extracted by first suspending 75 mg of the freeze-dried sample into 1.5 mL buffer A (25 mM MES buffer pH 6.5, 1 mM PMSF and 1 mM DTT). The suspensions were mixed intermittently for 15 min and centrifuged (20,000 × *g*; 10 min; 4 °C). The supernatant, which contains easily extractable proteins, was collected. The pellet was washed with 1.5 mL buffer A and then suspended in 1.5 mL buffer B (25 mM MES buffer pH 6.5, 1 mM PMSF, 1 mM DTT, 1 mM EDTA and 50 mM NaCl). The cells in the suspension were disrupted by a digital sonifier (Branson, Danbury, CT, USA) which was set at 30% amplitude, 4 times of 30 s intervals with 30 s periods between intervals. The cell debris was removed by centrifugation (20,000 × *g*; 10 min; 4 °C). The supernatant, containing pellet-associated proteins, was collected. During the extraction process, the samples were kept in ice-water bath.

2.3.5. Protein content

Protein content of the extracts was measured by mixing 20 μL of the sample or diluted sample with 200 μL of Bradford reagent (Bradford, 1976) obtained from Sigma–Aldrich. After 10 min incubation at room temperature, the absorbance was measured at 595 nm. A standard curve was prepared using bovine serum albumin (Sigma–Aldrich) dissolved in buffer A with a concentration of 0–300 μg/mL.

2.3.6. Analyses of enzyme activities

2.3.6.1. Glycosidases. To measure the activities of glycosidases, synthetic substrate p-nitrophenyl (PNP) glycosides were used. PNP-α-D-glucopyranoside, PNP-β-D-glucopyranoside, PNP-β-D-xylopyranoside and PNP-α-L-arabinofuranoside were obtained from Sigma–Aldrich. Glycosidase activity was measured by mixing 20 μL of protein extracts and 200 μL of 1 mM PNP-glycosides in a microtitre plate. The plate was incubated at 37 °C and the absorbance at 405 nm was measured every 3 min for 2 h. The concentration of p-nitrophenol at every time point was quantified using a standard curve (0–500 μM p-nitrophenol). The concentration of p-nitrophenol was then plotted against time, and the linear range was used to calculate enzyme activity, which was expressed in the amount of p-nitrophenol (nmol) released in 1 min by enzymes extracted from 1 mg dry digesta.

2.3.6.2. Polysaccharide-degrading enzymes. Some polysaccharides were treated before being used as substrates for analyses of enzyme activities. To be able to measure activities towards pectin backbone without the interference of the neutral side chains, high methyl esterified (HM) pectin and low methyl esterified (LM) pectin (C74 and C30, Copenhagen Pectin, Copenhagen, Denmark; Daas, Boxma, Hopman, Voragen, & Schols, 2001) were treated with single component enzyme preparations containing endoarabinanase, arabinofuranosidase and galactanase (Novozymes, Bagsvaerd, Denmark) to remove the neutral side chains (pH 5.0 ± 0.1; 24 h; 35 °C). After incubation, the enzymes were inactivated by boiling for 10 min. The pectins were precipitated in 70% (v/v) ethanol in water and filtered, followed by washing with 80% (v/v) ethanol and subsequently with acetone. After drying, the pectins were milled (MM2000, Retsch) to obtain fine powder. Oat spelt xylan (Sigma–Aldrich) was washed with water to remove soluble materials. After centrifugation (1500 × *g*; 5 min; room temperature), the insoluble part was washed in 96% (v/v) ethanol and dried.

In total, 10 polysaccharides were used as substrates to analyse enzyme activities in the enzyme mixture. Soluble potato starch (Sigma–Aldrich), soluble wheat arabinoxylan (medium viscosity, Megazyme), barley mixed-linkage β-glucan (Megazyme), carboxymethyl cellulose (CMC, sodium salt, medium viscosity, Sigma–Aldrich), soluble soy polysaccharide (SSPS, Soyafibe-S-DA-100, Fuji Oil Co., Ibaraki, Japan), locust bean gum (SKW Biosystems, Rubi, Spain), HM and LM pectins without side chains, were dissolved in 25 mM MES buffer pH 6.5 at 2 mg/mL. The substrate solutions were heated at 100 °C for 5 min with occasional mixing for maximum dissolution. The pH of the solutions were adjusted to 6.5 ± 0.1 if necessary using 4 N NaOH solution. The insoluble substrates oat spelt xylan and cellulose (Avicel PH105, Serva Feinbiochemica, Heidelberg, Germany) were weighed directly into the tubes and MES buffer (25 mM, pH 6.5) was added to reach a concentration of 2 mg/mL.

Protein extracts were added into the substrate solution or suspension at a ratio of 1:10 (v/v). The mixture was incubated for 30 min at 37 °C, followed by enzyme inactivation by boiling for 5 min. Substrate blanks was prepared by substituting the enzyme extracts with MES buffer (25 mM, pH 6.5) and enzyme blanks was prepared by substituting the substrate with MES buffer (25 mM, pH 6.5). The amount of reducing sugars in the enzyme digests and blanks was quantified using 4-hydroxybenzoic acid hydrazide (PAHBAH) method adapted for microtitre plate (Kühnel et al., 2010). Standard curves (0–250 μg/mL) of monosugars were used for quantification. Glucose was used as a standard for starch, mixed-linkage β-glucans, CMC and cellulose digests; xylose was used as a standard for wheat arabinoxylan and oat spelt xylan digests; galactose, galacturonic acid and mannose were used as standards for SSPS, pectins and locust bean gum digests, respectively. The enzyme activity was expressed as the amount of reducing sugars (nmol) released in 1 min by enzymes extracted from one mg dry digesta.

2.4. Statistical analyses

Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA). Comparison between the two treatments at different parts of the large intestine was performed using *t*-test (PROC TTEST). Comparison among the different parts of large intestine in one treatment was performed using mixed-effect model (PROC MIXED) with parts of the large intestine as a fixed effect and individual pigs as a random effect. Differences between different parts of the large intestine was analysed using LSMEANS with Tukey–Kramer adjustment for unbalanced data. The confidence level (alpha) was set at 0.10. In the clustering analysis (PROC CLUSTER), complete linkage method was used.

3. Results and discussion

3.1. Sugar composition

The main monosaccharides in the NSPs of both diets were arabinose, xylose, and glucose, which composed more than 80% of the total NSPs (Table 2). The NSPs in the diet originated from wheat and barley. The main NSPs in both cereals are arabinoxylan, cellulose and mixed-linkage β-glucans (Englyst, Bingham, Runswick, Collinson, & Cummings, 1989). Only 25% of NSPs in DS diet and 23%

Table 2
Sugar composition of total non-starch polysaccharides (NSPs) in the experimental diets.

	g/100 g dry weight								
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total
Digestible starch diet									
Soluble NSPs	0.0	0.0	0.3	0.5	0.4	0.1	0.5	0.1	1.9
Insoluble NSPs	0.0	0.0	0.9	2.1	0.3	0.1	2.0	0.2	5.6
Total NSPs	0.0	0.0	1.2	2.5	0.7	0.2	2.5 (0.7)[a]	0.3	7.5
Resistant starch diet									
Soluble NSPs	0.0	0.0	0.3	0.3	0.4	0.1	0.4	0.1	1.6
Insoluble NSPs	0.0	0.0	1.0	2.0	0.4	0.1	1.9	0.2	5.5
Total NSPs	0.0	0.0	1.2	2.3	0.7	0.3	2.3 (0.7)[a]	0.3	7.2

NSPs: non-starch polysaccharides, Rha: rhamnose, Fuc: fucose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: uronic acids.
[a] Numbers in brackets are mixed-linkage β-glucans.

Table 3
Sugar composition of digesta from different parts of pig large intestine.

Diet	Part	g/100 g dry weight											
		Arabinose		Xylose		Glucose						Total	
						Starch		β-Glucan		Cellulose[#]			
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DS	Cae	4.4	0.6	8.2	1.5	1.7a[*]	0.9	0.08a[*]	0.03	9.9a	1.2	29a[*]	3.8
	pCol	4.4	0.6	8.1	1.6	0.8b	0.4	0.03b	0.01	10.2a	1.8	27ab	4.3
	pmCol	4.3	0.5	7.8	1.6	0.6b[*]	0.4	n.a.		9.2ab	1.4	26abc	3.3
	dmCol	3.6[*]	0.2	6.1[*]	0.5	0.5b	0.1	n.a.		7.3b[*]	0.6	21c[*]	1.4
	dCol	3.8[*]	0.3	6.5	0.8	0.6b	0.2	n.a.		7.5b[*]	0.4	22bc[*]	1.4
RS	Cae	4.2	0.4	8.0	1.4	15.1a[*]	5.2	0.64a[*]	0.36	10.6	2.7	43a[*]	8.2
	pCol	4.0	0.7	7.0	0.7	3.2b	2.1	0.12b	0.14	7.7	5.5	30b	3.3
	pmCol	4.5	0.4	7.5	0.8	1.1b[*]	0.3	n.a.		9.9	1.4	28b	2.4
	dmCol	4.4[*]	0.5	7.3[*]	1.1	0.7b	0.3	n.a.		9.1[*]	1.3	26b[*]	3.7
	dCol	4.5[*]	0.5	8.0	1.5	0.8b	0.2	n.a.		9.6[*]	1.4	28b[*]	3.7

Data with different letters within a column within a treatment are statistically different (P-value < 0.10). DS: digestible starch, RS: resistant starch. Cae: caecum, pCol: proximal colon, pmCol: proximal mid-colon, dmCol: distal mid-colon, dCol: distal colon. n.a.: not analysed.
[*] Statistically different (*P*-value < 0.10) results between treatments.
[#] Cellulose was calculated from the total glucose content after being corrected for starch and β-glucan. This fraction may also contain other glucose-containing NSPs.

of NSPs in RS diet were soluble. The glucose moieties in soluble NSPs were mainly from mixed-linkage β-glucans.

For the large intestinal digesta, only results of arabinose, xylose, and glucose are presented (Table 3) as other sugars were present in minor amounts. Starch and mixed-linkage β-glucans were measured separately, and glucose moieties from cellulose and other glucose-containing NSPs were calculated from the total glucose content in the digesta.

In the Cae, the amount of starch and its degradation products was higher for RS-fed pigs than for DS-fed pigs (*P*-value < 0.10). Starch and its degradation products that are not absorbed in the small intestine and were present in the caecum are considered as resistant starch. In the pCol of RS-fed pigs, the starch content was lower than that in Cae (*P*-value < 0.10), and was not statistically different from the starch content in the pCol of DS-fed pigs (*P*-value = 0.17). This indicated that resistant starch was mainly utilised in the Cae. Rapid utilisation of resistant starch in the caecum of pigs was also reported by Govers, Gannon, Dunshea, Gibson, and Muir (1999).

β-Glucans in the Cae of DS-fed pigs and RS-fed pigs were 0.8% and 5.7% of total NSP glucose present, respectively. In the diet, β-glucans composed 28-30% of the glucose from NSP. The low percentages of β-glucans in the digesta show that β-glucans were utilised in the Cae for both diets. This result was in agreement with previous report which stated that β-glucans were utilised in the proximal parts of the large intestine (Knudsen, Jensen, & Hansen, 1993). Nevertheless, the lower β-glucan content in the Cae of DS-fed pigs compared to that of RS-fed pigs indicated that the utilisation of β-glucans was slightly delayed when resistant starch was present in the diet.

The content of cellulose and other glucose-containing NSPs were higher for RS-fed pigs in the distal parts of the large intestine. This might suggest higher utilisation of cellulose and glucose-containing NSPs in DS-fed pigs. On the other hand, it may also indicate higher microbial count in RS-fed pigs, because it has been reported that the bacterial fraction in human faeces contains considerable amount of glucose (Cabotaje, Lopez-Guisa, Shinnick, & Marlett, 1990).

Arabinose and xylose concentrations in the large intestine were not statistically different between the two treatments except for the distal parts of the colon. This may indicate that the utilisation of arabinoxylan is more extensive in the DS-fed pigs. Nonetheless, without an indigestible marker to calculate the apparent digestibility, this effect cannot be quantified.

The composition of soluble sugars in the digesta was measured separately (Table 4). The glucose concentration in the soluble part of Cae was higher for RS-fed pigs than for DS-fed pigs (*P*-value < 0.10). This was expected, because as resistant starch was utilised, it is degraded into smaller, soluble molecules, prior to full utilisation of the glucose. Solubilisation of feed components due to enzyme activity has been used for evaluating the total tract digestibility (Boisen & Fernández, 1997). It has also been reported that some gut microbes, such as *Bacteroides thethaiotamicron*, has enzymes capable of degrading starch molecules to smaller molecules which are then absorbed into the cell for further utilisation (Shipman, Cho, Siegel, & Salyers, 1999).

Along the large intestine, glucose concentration in the soluble part of the digesta was higher for RS-fed pigs than for DS-fed pigs. As discussed previously for total glucose content, soluble glucose in distal parts of the large intestine may originate from

Table 4
Sugar composition of soluble fraction of digesta from different parts of pig large intestine.

Diet	Part	g/100 g dry weight							
		Arabinose		Xylose		Glucose		Total	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
DS	Cae	0.03a*	0.00	0.03*	0.01	0.33a*	0.12	0.64a*	0.19
	pCol	0.02b	0.00	0.02	0.01	0.13b	0.02	0.37b	0.02
	pmCol	0.02ab*	0.00	0.02	0.00	0.07b*	0.01	0.32b*	0.03
	dmCol	0.02ab	0.00	0.02*	0.00	0.06b*	0.00	0.29b*	0.01
	dCol	0.02ab	0.01	0.02	0.01	0.05b*	0.01	0.29b*	0.06
RS	Cae	0.10a*	0.06	0.17a*	0.12	2.86a*	0.80	3.69a*	1.00
	pCol	0.02b	0.01	0.03b	0.02	0.76b	0.58	1.34b	0.63
	pmCol	0.01b*	0.00	0.02b	0.01	0.26b*	0.08	0.76b*	0.15
	dmCol	0.01b	0.00	0.01b*	0.00	0.20b*	0.06	0.70b*	0.17
	dCol	0.02b	0.02	0.02b	0.02	0.18b*	0.04	0.61b*	0.14

Data with different letters within a column within a treatment are statistically different (P-value < 0.10). DS: digestible starch, RS: resistant starch. Cae: caecum, pCol: proximal colon, pmCol: proximal mid-colon, dmCol: distal mid-colon, dCol: distal colon.
* Statistically different (P-value < 0.10) results between treatments.

Table 5
Extractable protein content in the large-intestinal digesta of pigs (μg/mg dry weight).

Diet	Part	Easily extracted proteins		Pellet associated proteins		Total extractable proteins	
		Mean	SD	Mean	SD	Mean	SD
DS	Cae	9.2a*	0.9	20.7a	1.6	29.9a	2.4
	pCol	7.2b*	0.7	16.0a*	3.2	23.2b*	3.4
	pmCol	5.3c	1.4	7.3b*	3.3	12.6c*	4.6
	dCol	2.4d*	0.5	5.6b*	3.2	8.0c*	3.7
RS	Cae	10.6a*	0.4	22.9	9.5	33.5ab	9.2
	pCol	10.5a*	1.7	28.1*	6.3	38.6a*	5.1
	pmCol	7.5a	2.4	26.7*	3.5	34.3ab*	5.6
	dCol	4.0b*	0.5	20.1*	2.4	24.1b*	2.9

Data with different letters within a column within a treatment are statistically different (P-value < 0.10). DS: digestible starch, RS: resistant starch, Cae: caecum, pCol: proximal colon, pmCol: proximal mid-colon, dmCol: distal mid-colon, dCol: distal colon.
* Statistically different (P-value < 0.10) results between treatments.

the content of microbial cells. This was also supported by the high amount of pellet-associated proteins, which is presented in Section 3.2.

The concentrations of soluble arabinose and xylose in Cae were higher for RS-fed pigs than for DS-fed pigs (Table 4, P-value < 0.10), although there was no difference in total arabinose and xylose contents between treatments (Table 3). However, the soluble arabinose and xylose represented only 0.5% (DS) and 2.3% (RS) of total arabinose and xylose present in Cae. Compared to the diets, in which 19.8% (DS) and 17.2% (RS) of the arabinose and xylose was soluble, the low percentages in the Cae shows that soluble arabinoxylan was utilised in Cae irrespective of the presence of resistant starch in the diet, but the utilisation was lower in the presence of resistant starch. These results further substantiate a previous study (Walker et al., 2011), which did not present data on monosaccharide constituents.

In summary, results of sugar composition showed that resistant starch was mainly fermented in Cae. When resistant starch was present in Cae, the utilisation of β-glucans and soluble arabinoxylan was delayed, indicating that the microbiota preferred to utilise resistant starch than the other dietary fibres. Preferred utilisation of one dietary fibre over the other by human microbiota was previously described for ispaghula husk over cellulose (Prynne & Southgate, 1979). The results also indicated that arabinoxylan was utilised more extensively in DS-fed pigs than in RS-fed pigs. However, without an indigestible marker in the diet, the apparent digestibility could not be quantified. For further assessment of the effect of resistant starch on the degradation of NSPs, enzymes in the digesta samples were extracted and their activities were measured.

3.2. Extractable proteins

Enzymatic proteins were extracted from the digesta, together with other proteins. The total extractable proteins consisted of easily extractable proteins and pellet-associated proteins, which was solubilised by ultrasonication (Table 5). Table 5 shows that more than 50% of the total protein was pellet-associated. Total extractable protein in the Cae did not differ between treatments, suggesting that the presence of resistant starch did not influence the digestion and absorption of proteins in the small intestine. In the colon, the level of these pellet-associated proteins was higher for the RS-fed pigs than for the DS-fed pigs (P-value < 0.10). These results suggest that the increase in pellet-associated proteins by resistant starch in the diet might be caused by the increase of microbial growth. Resistant starch is a fermentable fibre, and fermentable fibres have been shown to be able to increase faecal bacterial mass (Stephen & Cummings, 1980).

Along the large intestine, total extractable protein content in the digesta of DS-fed pigs declined between Cae and pCol and between pCol and pmCol. In contrast, the total extractable protein content in the digesta of RS-fed pigs only declined in the distal parts of the colon. The decline of total extractable protein content could be caused by degradation and utilisation of enzymes and proteins from dead microbial cells. *In vitro*, it has been shown that when carbohydrate supply was limited, protein fermentation by human faecal microbiota was more evident (Macfarlane, Quigley, Hopkins, Newton, & Macfarlane, 1998). Hence, it could be suggested that for RS-fed pigs, the stable level of the protein content in the digesta along the colon indicates that more microbiota colonised the distal colon than in DS-fed pigs.

Table 6
Enzyme activity in large intestinal digesta of pigs (nmol/mg dry digesta/min).

Diet	Part	Glycosidases				Polysaccharide-degrading enzymes								
		α-glc	β-glc	β-xyl	α-ara	wax	β-glucans	cmc	hmp	lmp	lbg	ssp	sta	osx	cel
DS	Cae														
	Mean	1.4a	2.6a*	1.6a*	5.2a*	3.7a	15.0a*	0.8	2.1	2.2	1.7	6.2	31.1a*	1.6	0.5
	SD	0.9	1.3	0.6	2.0	0.8	5.3	0.8	1.2	1.4	1.9	3.0	11.2	1.3	0.7
	pCol														
	Mean	0.8ab	1.9ab	1.5a*	3.4ab*	2.8a	9.6b	0.7	2.1	2.0	1.7	3.2	12.7b	1.2	0.6
	SD	0.1	0.9	0.2	1.1	0.5	1.4	0.8	1.2	1.2	1.9	3.1	0.9	0.8	0.8
	pmCol														
	Mean	0.5b	0.9b	1.0ab*	1.3bc	1.6b	3.1c	0.7	1.9	2.0	1.6	2.5	9.3b	0.7	0.3
	SD	0.1	0.6	0.2	0.4	0.5	0.9	0.8	1.2	1.3	1.9	2.8	0.8	0.8	0.4
	dCol														
	Mean	0.2b	0.4b	0.6b*	0.8c	1.4b	2.1c	0.9	2.2	2.4	1.1	1.4	7.8b*	0.6	0.6
	SD	0.1	0.2	0.2	0.2	0.8	1.9	1.0	1.0	1.1	1.5	2.3	1.9	0.4	0.7
RS	Cae														
	Mean	1.3ab	0.9	0.6ab	2.4a	2.7	8.0ab	1.5	1.4	2.6	2.0	10.7a	55.5a	2.0	0.7
	SD	0.6	0.5	0.2	1.1	1.3	1.6	0.8	0.3	0.9	0.9	7.7	8.3	2.2	0.9
	pCol														
	Mean	1.6a	0.9	0.9a	2.0a	2.9	9.7a	0.8	2.2	2.9	1.7	3.4ab	27.6b	1.6	1.0
	SD	1.0	0.2	0.1	0.5	0.6	2.3	1.4	1.4	1.7	2.9	1.1	10.2	1.2	1.7
	pmCol														
	Mean	0.8ab	0.8	0.7ab	1.2ab	2.0	6.7ab	0.7	2.4	2.5	1.5	4.2ab	18.5bc	1.1	0.3
	SD	0.6	0.2	0.2	0.3	0.6	3.4	0.8	1.4	1.9	1.8	3.2	8.4	1.0	0.3
	dCol														
	Mean	0.4b	0.4	0.4b	0.7b	1.9	3.2b	0.8	2.6	2.4	1.0	1.6b	12.3c	1.0	0.4
	SD	0.2	0.1	0.1	0.2	0.9	1.5	0.9	1.3	1.7	1.6	2.5	2.9	0.8	0.5

Data with different letters within a column within a treatment are statistically different (P-value < 0.10). DS: digestible starch, RS: resistant starch, Cae: caecum, pCol: proximal colon, pmCol: proximal mid-colon, dmCol: distal mid-colon, dCol: distal colon, α-glc: α-glucopyranosidase, β-glc: β-glucopyranosidase, β-xyl: β-xylopyranosidase, α-ara: α-arabinofuranosidase, wax: wheat arabinoxylan, cmc: carboxymethyl cellulose, hmp: high methylated pectin, lmp: low methylated pectin, lbg: locust bean gum, ssp: soluble soy polysaccharide, sta: starch, osx: oat spelt xylan, cel: cellulose.

* Statistically different (P-value < 0.10) results between treatments.

A part of the protein in the digesta will certainly be polysaccharide-degrading enzymes. Because most of the microbial polysaccharide-degrading enzymes are cell-associated (Macfarlane et al., 1998), the amount of these enzymes were expected to increase as the microbial growth was stimulated by resistant starch in the diet.

3.3. Enzyme activities in the digesta

In an *in vitro* model, the enzymes produced by human faecal microbiota were influenced by the substrates present (Macfarlane et al., 1998). Therefore, a wide spectrum of substrates was used, including those that were not present in the diet. The enzyme activities in the digesta towards 14 different substrates are summarised in Table 6.

Total α-glucopyranosidase activity was not influenced by the presence of resistant starch in the diet (P-value = 0.89), but the activity towards starch in the Cae was higher for RS-fed pigs than for DS-fed pigs. This may show that the presence of resistant starch in the Cae stimulated the growth of microbiota that can produce and maintain the level of starch-degrading enzymes.

Contrary to enzyme activities related to starch degradation, the activity of β-glucopyranosidase as well as the activity towards barley mixed-linkage β-glucans in the Cae was higher for DS-fed pigs than for RS-fed pigs. This might show that due to the low level of starch in the Cae of DS-fed pigs, the microbiota used mixed-linkage β-glucans as an alternative substrate by producing β-glucanases and β-glycosidases. Mixed-linkage β-glucan, therefore, was more extensively utilised in the Cae of DS-fed pigs than in RS-fed pigs (Section 3.1). Further along the large intestine, the activity towards mixed-linkage β-glucans in DS-fed pigs declined steadily, whereas in RS-fed pigs the activity was maintained until the pmCol. In this location, the activity towards mixed-linkage β-glucans was slightly higher for RS-fed pigs than for DS-fed pigs (P-value = 0.12).

The activities of β-xylopyranosidase and α-arabinofuranosidase were higher in the Cae of DS-fed pigs than of RS-fed pigs. This may explain the higher utilisation of soluble arabinoxylan by the microbiota in DS-fed pigs than in RS-fed pigs (Section 3.1). The activity towards wheat arabinoxylan, which is an ensemble of endoxylanase, β-xylopyranosidase and α-arabinofuranosidase activities, however, did not differ between treatments (P-value = 0.27).

It was observed that enzyme activities related to the degradation of starch, mixed-linkage β-glucans and arabinoxylans, which represented polysaccharides in the diet, declined along the large intestine. This suggested that the production of these enzymes was stimulated in the Cae when the substrates were abundant. Further along the large intestine, the substrates were depleted, enzyme production was no longer stimulated, and the available enzymes were utilised by the microbiota together with other proteins.

Unlike the activities towards starch and soluble NSPs, enzyme activities towards insoluble NSPs, such as xylan and cellulose, were low, and did not differ between diets (P-value > 0.75). It was reported that faecal microbiota from pigs has a lower ability to utilise insoluble NSPs than soluble NSPs (Williams, Mikkelsen, le Paih, & Gidley, 2011). It has also been reported that resistant starch did not influence the digestibility of insoluble NSPs in humans (Walker et al., 2011), but in these studies the NSPs were not further specified.

For other soluble NSPs that were not present in the diet, such as HM and LM pectins with removed side chains and locust bean gum, the degrading-enzyme activity along the large intestine was constantly low. There was no difference between diets and no difference between different parts of the large intestine. This may show that despite the absence of the substrates in the diet, the microbiota in the large intestine produced enzymes, which are able to degrade these substrates, in low amounts. If the substrate becomes available, the growth of substrate-specific bacteria might be stimulated, thereby the enzyme production might be increased to facilitate the utilisation of the substrates.

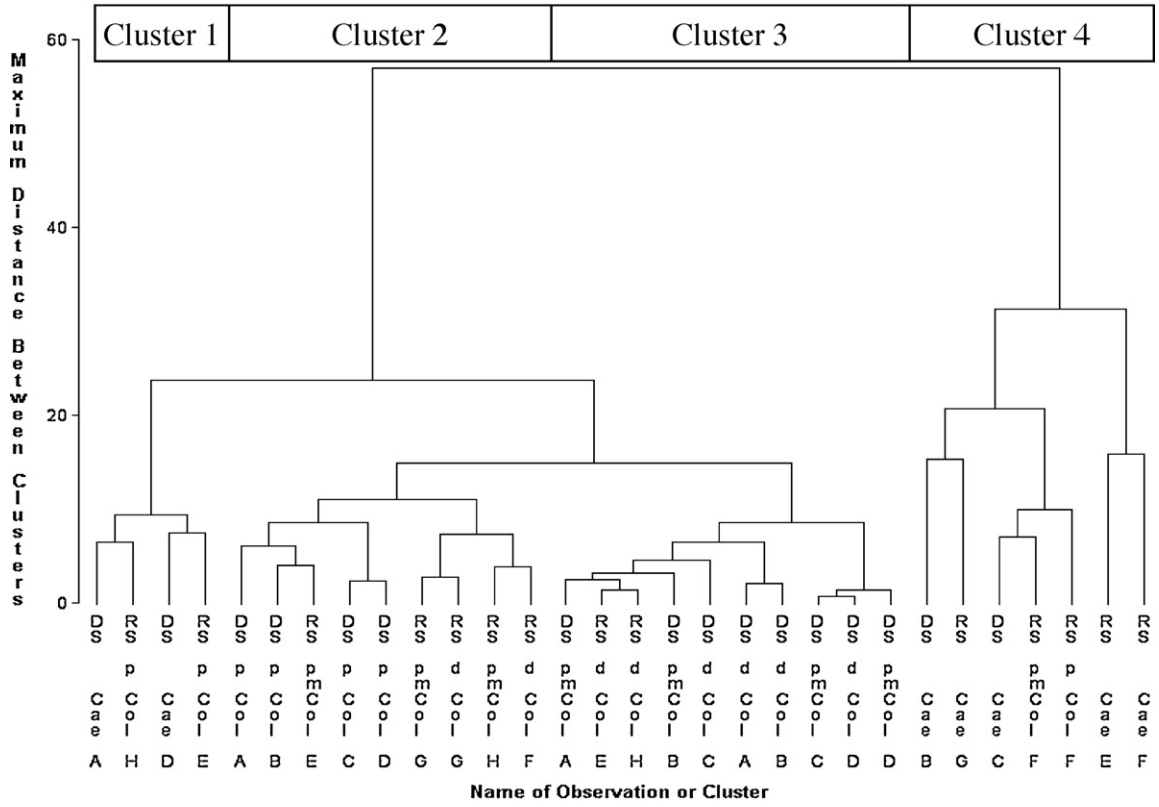

Fig. 1. Cluster analysis of pig digesta samples based on enzyme activities towards 14 substrates. DS: digestible starch diet, RS: resistant starch diet, Cae: caecum, pCol: proximal colon, pmCol: proximal mid-colon, dCol: distal colon. A–D = pigs fed with DS diet, E–H = pigs fed with RS diet.

SSPS is a substrate that was not present in the diet, but it was degraded at a similar level as wheat arabinoxylan. SSPS consist of pectic rhamnogalacturonan backbone, with side chains of α-arabinans and β-galactans (Nakamura, Furuta, Maeda, Nagamatsu, & Yoshimoto, 2001). β-Galactans were known to be present in wheat endosperm in the form of arabinogalactan (Fincher, Sawyer, & Stone, 1974). Therefore, the capability of the enzyme mixture to degrade SSPS may indicate that the microbiota in the large intestine of pigs was able to utilise arabinogalactan present in the diet.

At the distal part of the large intestine, total protein content was increased by three-fold when resistant starch was present. In contrast, there was no difference between treatments for most of the enzyme activities (P-value > 0.35), except for starch-degrading enzymes. This might mean that resistant starch stimulated the growth of selective microbiota, and that the microbiota stimulated by resistant starch produced starch-degrading enzymes, but lack the ability to produce degrading enzymes for other polysaccharides.

In order to summarise the findings on the enzyme activities, the results were compiled together and subjected to cluster analysis. The samples formed four clusters (Fig. 1). All Cae samples from RS-fed pigs were in a distinct cluster, together with half of the Cae samples from DS-fed pigs (Cluster 4). Cluster 1 consists of samples from the pCol of RS-fed pigs and the other half of the Cae samples from DS-fed pigs. Further, Cluster 2 consists of samples from the pmCol and some samples from the dCol of RS-fed pigs and samples from the pCol of DS-fed pigs. Cluster 3 consists of samples from the pmCol and dCol of DS-fed pigs and some samples from the dCol of RS-fed pigs.

This clustering analysis confirms and clearly visualises that the presence of resistant starch changed the enzyme profile in the large intestine. The enzyme profile in a certain part of the large intestine of RS-fed pigs resembles its more proximal counterpart of DS-fed pigs. These results suggest that in RS-fed pigs, the microbiota in the mid-colon was more active in degrading polysaccharides than in DS-fed pigs. Polysaccharide degradation at the distal parts of the large intestine is regarded as beneficial for health (Topping & Clifton, 2001). This experiment, therefore, showed that although resistant starch itself is mainly utilised in the caecum, it might positively affect colon health by delaying the degradation of other dietary fibres.

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References

Abell, G. C. J., Cooke, C. M., Bennett, C. N., Conlon, M. A., & McOrist, A. L. (2008). Phylotypes related to *Ruminococcus bromii* are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. *FEMS Microbiology Ecology*, *66*(3), 505–515.

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews*, *70*(2), 567–590.

Boisen, S., & Fernández, J. A. (1997). Prediction of the total tract digestibility of energy in feedstuffs and pig diets by in vitro analyses. *Animal Feed Science and Technology*, *68*(3–4), 277–286.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248–254.

Cabotaje, L. M., Lopez-Guisa, J. M., Shinnick, F. L., & Marlett, J. A. (1990). Neutral sugar composition and gravimetric yield of plant and bacterial fractions of feces. *Applied and Environmental Microbiology*, *56*(6), 1786–1792.

Cummings, J. H., Edmond, L. M., & Magee, E. A. (2004). Dietary carbohydrates and health: Do we still need the fibre concept? *Clinical Nutrition Supplements*, *1*(2), 5–17.

Daas, P. J. H., Boxma, B., Hopman, A. M. C. P., Voragen, A. G. J., & Schols, H. A. (2001). Nonesterified galacturonic acid sequence homology of pectins. *Biopolymers*, *58*(1), 1–8.

De Schrijver, R., Vanhoof, K., & Vande Ginste, J. (1999). Nutrient utilization in rats and pigs fed enzyme resistant starch. *Nutrition Research*, *19*(9), 1349–1361.

Dongowski, G., Jacobasch, G., & Schmiedl, D. (2005). Structural stability and prebiotic properties of resistant starch type 3 increase bile acid turnover and lower secondary bile acid formation. *Journal of Agricultural and Food Chemistry*, *53*(23), 9257–9267.

Englyst, H. N., Bingham, S. A., Runswick, S. A., Collinson, E., & Cummings, J. H. (1989). Dietary fibre (non-starch polysaccharides) in cereal products. *Journal of Human Nutrition and Dietetics*, *2*(4), 253–271.

Englyst, H. N., & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas–liquid chromatography of constituent sugars as alditol acetates. *Analyst*, *109*(7), 937–942.

Englyst, H. N., Quigley, M. E., & Hudson, G. J. (1994). Determination of dietary fibre as non-starch polysaccharides with gas–liquid chromatographic, high-performance liquid chromatographic or spectrophotometric measurement of constituent sugars. *Analyst*, *119*(7), 1497–1509.

Fincher, G. B., Sawyer, W. H., & Stone, B. A. (1974). Chemical and physical properties of an arabinogalactan peptide from wheat endosperm. *Biochemical Journal*, *139*(3), 535–545.

Govers, M. J. A. P., Gannon, N. J., Dunshea, F. R., Gibson, P. R., & Muir, J. G. (1999). Wheat bran affects the site of fermentation of resistant starch and luminal indexes related to colon cancer risk: A study in pigs. *Gut*, *45*(6), 840–847.

Knudsen, K. E. B., Jensen, B. B., & Hansen, I. (1993). Digestion of polysaccharides and other major components in the small and large intestine of pigs fed on diets consisting of oat fractions rich in β-D-glucan. *British Journal of Nutrition*, *70*(02), 537–556.

Kühnel, S., Hinz, S. W. A., Pouvreau, L., Wery, J., Schols, H. A., & Gruppen, H. (2010). Chrysosporium lucknowense arabinohydrolases effectively degrade sugar beet arabinan. *Bioresource Technology*, *101*(21), 8300–8307.

Lesmes, U., Beards, E. J., Gibson, G. R., Tuohy, K. M., & Shimoni, E. (2008). Effects of resistant starch type III polymorphs on human colon microbiota and short chain fatty acids in human gut models. *Journal of Agricultural and Food Chemistry*, *56*(13), 5415–5421.

Louis, P., Scott, K. P., Duncan, S. H., & Flint, H. J. (2007). Understanding the effects of diet on bacterial metabolism in the large intestine. *Journal of Applied Microbiology*, *102*(5), 1197–1208.

Macfarlane, S., Quigley, M. E., Hopkins, M. J., Newton, D. F., & Macfarlane, G. T. (1998). Polysaccharide degradation by human intestinal bacteria during growth under multi-substrate limiting conditions in a three-stage continuous culture system. *FEMS Microbiology Ecology*, *26*(3), 231–243.

Martin, L. J. M., Dumon, H. J. W., & Champ, M. M. J. (1998). Production of short-chain fatty acids from resistant starch in a pig model. *Journal of the Science of Food and Agriculture*, *77*(1), 71–80.

Martínez, I., Kim, J., Duffy, P. R., Schlegel, V. L., & Walter, J. (2010). Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. *PLoS ONE*, *5*(11).

Martínez-Puig, D., Castillo, M., Nofrarias, M., Creus, E., & Pérez, J. F. (2007). Long-term effects on the digestive tract of feeding large amounts of resistant starch: A study in pigs. *Journal of the Science of Food and Agriculture*, *87*(11), 1991–1999.

Michalet-Doreau, B., Fernandez, I., & Fonty, G. (2002). A comparison of enzymatic and molecular approaches to characterize the cellulolytic microbial ecosystems of the rumen and the cecum. *Journal of Animal Science*, *80*(3), 790–796.

Nakamura, A., Furuta, H., Maeda, H., Nagamatsu, Y., & Yoshimoto, A. (2001). Analysis of structural components and molecular construction of soybean soluble polysaccharides by stepwise enzymatic degradation. *Bioscience, Biotechnology and Biochemistry*, *65*(10), 2249–2258.

Nugent, A. P. (2005). Health properties of resistant starch. *Nutrition Bulletin*, *30*(1), 27–54.

Prynne, C. J., & Southgate, D. A. T. (1979). The effects of a supplement of dietary fibre on faecal excretion by human subjects. *British Journal of Nutrition*, *41*(3), 495–503.

Regmi, P. R., Metzler-Zebeli, B. U., Gänzle, M. G., van Kempen, T. A. T. G., & Zijlstra, R. T. (2011). Starch with high amylose content and low *in vitro* digestibility increases intestinal nutrient flow and microbial fermentation and selectively promotes Bifidobacteria in pigs. *The Journal of Nutrition*, *141*(7), 1273–1280.

Rideout, T. C., Liu, Q., Wood, P., & Fan, M. Z. (2008). Nutrient utilisation and intestinal fermentation are differentially affected by the consumption of resistant starch varieties and conventional fibres in pigs. *British Journal of Nutrition*, *99*(05), 984–992.

Shipman, J. A., Cho, K. H., Siegel, H. A., & Salyers, A. A. (1999). Physiological characterization of SusG, an outer membrane protein essential for starch utilization by *Bacteroides thetaiotaomicron*. *Journal of Bacteriology*, *181*(23), 7206–7211.

Stephen, A. M., & Cummings, J. H. (1980). Mechanism of action of dietary fibre in the human colon. *Nature*, *284*(5753), 283–284.

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. *Physiological Reviews*, *81*(3), 1031–1064.

Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., et al. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME Journal*, *5*(2), 220–230.

Williams, A. G., & Withers, S. E. (1991). Effect of ciliate protozoa on the activity of polysaccharide-degrading enzymes and fibre breakdown in the rumen ecosystem. *Journal of Applied Microbiology*, *70*(2), 144–155.

Williams, B. A., Mikkelsen, D., le Paih, L., & Gidley, M. J. (2011). In vitro fermentation kinetics and end-products of cereal arabinoxylans and (1,3;1,4)-β-glucans by porcine faeces. *Journal of Cereal Science*, *53*(1), 53–58.