

The background is a blue gradient with various white geometric shapes and lines. There are several prominent chains of shapes: a chain of hexagons at the top, a chain of pentagons below it, a chain of ovals in the middle, and a chain of rounded rectangles at the bottom. There are also scattered lines and other polygons throughout the background.

Monitoring the degradation of individual dietary fibres in pig models

Melliana Christina Jonathan

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Thesis

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Abstract

In this thesis, the degradation of dietary fibres in the gastrointestinal tract, especially in the large intestine, is monitored using *in vitro* and *in vivo* studies. First, an *in vitro* method to simulate the conditions in the mouth, stomach and small intestine was adapted for food products, which were used in an *in vivo* satiation study as well. Alginate, a dietary fibre that is able to form a gel under stomach conditions, was found to be more satiating than cellulose and guar gum. Next, *in vitro* fermentation were performed in addition to *in vivo* studies using pigs as models for humans. The rates and products of dietary fibre fermentation depend on the constituent monosaccharide and linkage compositions, degree of polymerisation and molecular conformation of the dietary fibres, as well as on the adaptability of the microbiota to the dietary fibres. The dietary fibres investigated in the *in vivo* pig studies included resistant starch, alginate and non-starch polysaccharides (NSPs) from other feed components. The large intestinal digesta and faecal samples were analysed to study how dietary fibres are degraded in the large intestine. Resistant starch was found to be preferred by the microbiota over NSPs. Hence, the utilisation of NSPs was delayed in the presence of resistant starch. The alginate used in the study was not fully utilised by the microbiota and more than 40 % (w/w) of the alginate intake was excreted in the faeces. The degradation products of alginate included an insoluble alginate fraction with increased guluronic acid content compared to the parental alginate. In addition, alginate oligosaccharides were formed. The time needed by the microbiota to adapt to different dietary fibres varied. For resistant starch, two weeks of adaptation was sufficient, but more than 39 days was required to adapt to alginate. The fermentability differences among diverse dietary fibres led to the conclusion that consumption of a changing diet containing various dietary fibres may ensure that fermentation occurs throughout the colon.

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Chapter 1

General Introduction

1.1. The project

Amongst many other health benefits, dietary fibres are recognized to be potential for preventing obesity (1). Studies about dietary fibres related to obesity are conducted through epidemiological studies (2), or through experiments in which human subjects were given diets supplemented with dietary fibres (3). The observations during the supplementation include hunger or fullness feeling, body weight change, and food intake (3).

One of the mechanisms by which dietary fibres can prevent obesity is by inducing earlier satiation (meal termination) and prolonging satiety (4). Some mechanisms by which dietary fibres affect satiation and satiety are known (5), but the experimental results with fibre supplementation are contradictory (3, 5). It is considered that the inconsistencies of the results obtained in experiments involving dietary fibres are partly caused by the diversity of dietary fibres used and the lack of detailed characterization of the fibres used in the experiments (3).

A multidisciplinary project named 'Food, fibre and health – an integrated approach' was set up, which was aimed to explore the role and the mechanisms of dietary fibres in inducing satiation and prolonging satiety. This thesis is a part of the project, and aimed to investigate the changes undergone by different dietary fibres in the gastrointestinal tract (GIT) using *in vitro* and *in vivo* experiments.

1.2. Definition of dietary fibres

The definition of dietary fibres has changed over time, as new findings related to dietary fibres were revealed. The changes are recorded in detail in recent reviews (6, 7). In Europe, dietary fibres are currently defined as: 'Carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed; edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence; edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence (8).'

Although dietary fibres are defined as carbohydrate polymers, it was stated that lignin and other compounds can be included in dietary fibre, if they are closely associated with the carbohydrate polymers in plant cell walls and are analysed together with the dietary fibres.

1.3. Classification of dietary fibres

With the definition cited above, dietary fibres include a large variety of carbohydrates with various constituent monosaccharide compositions, molecular weights, physical properties and physiological effects. Dietary fibres are often classified based on their physical properties, especially based on their solubility in water and viscosity. Nevertheless, it has been pointed out that classification of carbohydrates based on the solubility in water is less functional, because solubility of dietary fibres can also be influenced by other factors, such as pH (9). It was suggested to classify carbohydrates based on their chemical structure or based on clear physiological properties (9). Figure 1–1 illustrates the classification of carbohydrates based on their digestibility in the upper GIT and their molecular size. It is shown in Figure 1–1 that dietary fibre consists of three groups: non-digestible oligosaccharides, resistant starch, and non-starch polysaccharides (NSPs). In the next paragraphs, these three main groups of dietary fibres are described in more detail.

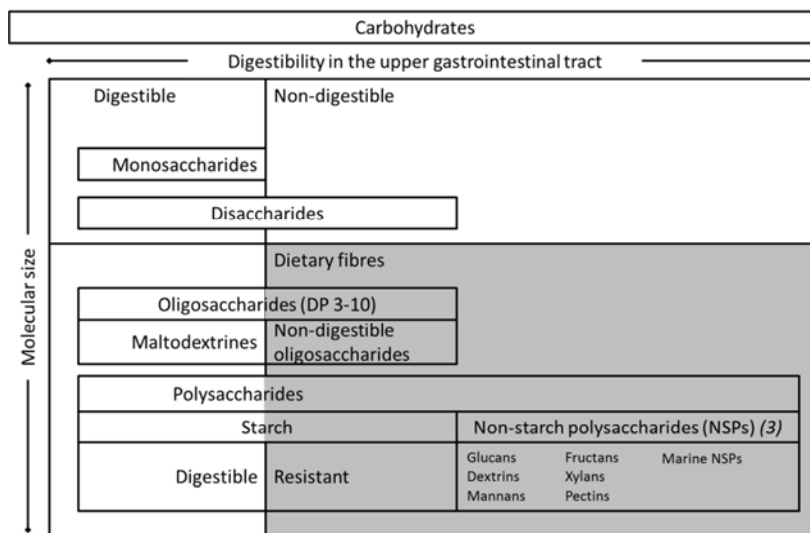


Figure 1–1. Chemical and physiological classification of carbohydrates. The classification is partially adapted from Cummings and Stephen (9). The grey box indicates the carbohydrates that are dietary fibres.

1.3.1. Non-digestible oligosaccharides

Oligosaccharides with degree of polymerisation (DP) 3-10 which are not digested in the upper GIT (NDOs) are explicitly included as dietary fibres in the definition stated by The American Association of Cereal Chemists (AACC) in 2001 (10). This AACC definition of dietary fibre coincides with the EC definition cited in Section 1.2, where it is stated that dietary fibres can have a degree of polymerisation (DP) of three or more. With such a broad definition, NDOs are very diverse, including degradation products of NSPs and some synthesized oligosaccharides (11), excluding mono- and disaccharides. Hence, non-digestible disaccharides, such as lactulose, are not dietary fibres.

The most well-known NDOs are fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), which have been shown to have prebiotic activity (12). Currently, other NDOs, including those produced from enzymatic digestion of different NSPs are emerging, such as xylo-oligosaccharides, alginate oligosaccharides, manno-oligosaccharides (11), and pectin oligosaccharides (13). Some of these oligosaccharides has already been commercialised (11, 14).

1.3.2. Resistant starch

Chemically, all starch is similar in having glucose residues linked by α -1,4 and α -1,6 linkages. Although human α -amylase can split the α -1,4 linkages, some starch is not absorbed in the small intestine and end up in the large intestine. The portion of starch that is not digested in the small intestine is named resistant starch.

Based on the causes why the starch is not digested in the upper gastrointestinal system, resistant starch is first divided into three groups (15): RS type 1, starch which is physically inaccessible because of entrapment in food; RS type 2, starch present in starch granules, and RS type 3: retrograded starch. The fourth group of resistant starch: RS type 4, chemically modified starches, was added later (16). Sources and properties of resistant starch have been reviewed in detail elsewhere (17, 18).

1.3.3. Non-starch polysaccharides

Non-starch polysaccharides (NSPs) are carbohydrates which are considered as dietary fibres ever since the term 'dietary fibre' was first introduced in 1953 (19, 20). Simply all polysaccharides besides starch belong to this group. NSPs, therefore, encompasses highly

diverse polysaccharides with various constituent monosaccharide composition and linkage types, as is exemplified in Table 1–1.

Table 1–1. Examples of the diversity of non-starch polysaccharides.

Dietary fibre class ¹	Examples of fibre variability	Main constituents	Sources
Cellulose		glucose	Plants
Mixed-linkage β -glucans (21)	β -1,3-1,4-glucan	glucose	Cereals
	β -1,3-1,6-glucan	glucose	Mushrooms, yeast
Hemicelluloses (22)	Arabinoxylans	arabinose, xylose	Cereals
	Glucuronoarabinoxylan	arabinose, xylose, glucuronic acid	Vegetative parts of grasses
	Xyloglucan	glucose, xylose	Plant cell wall of dicots and conifers
	Glucomannan Galactoglucomannan	mannose, glucose mannose, glucose, galactose	<i>Amorphophallus konjac</i> Plant cell wall of Gymnospermae
Pectic substances (23)	Homogalacturonan	galacturonic acid methyl-esterified galacturonic acid	Fruits and vegetables
	Complex pectin (rhamnogalacturonan)	galacturonic acid, rhamnose, arabinose, galactose	
Plant exudate gums	Arabic gum	arabinose, galactose, rhamnose, glucuronic acid	<i>Acacia senegal</i>
	Ghatti gum	arabinose, galactose, mannose, xylose, glucuronic acid, rhamnose	<i>Anogeissus latifolia</i>
	Karaya gum	galactose, rhamnose, galacturonic acid, glucuronic acid	<i>Sterculia spp.</i>
	Tragacanth gum	arabinose, galactose, galacturonic acid, xylose, rhamnose, fucose	<i>Astragalus spp.</i> , especially <i>A. gummifer</i>
Mucilages	Ispaghula husk (24)	xylose, arabinose, galacturonic acid, rhamnose	<i>Plantago ovata</i>
Endospermic leguminous seeds	Guar gum	mannose, galactose	Guar plant (<i>Cyamopsis tetragonolobus</i>)
	Locust bean gum	mannose, galactose	Carob tree (<i>Ceratoniasiliqua</i>)
Seaweed cell wall polysaccharides	Alginate	guluronic acid, mannuronic acid	Brown seaweed
	Carrageenan	galactose, anhydro galactose	Red seaweed
	Agar	galactose, anhydro galactose	Red seaweed
Chitin and chitosan (25)		glucosamine N-acetyl glucosamine	Fungi, crustaceans, insects
Microbial polysaccharides	Xanthan gum	glucose, glucuronic acid, mannose	<i>Xanthomonas campestris</i>
	Gellan gum	glucose, glucuronic acid, rhamnose	<i>Sphingomonas paucimobilis</i> (formerly <i>Pseudomonas elodea</i>) (26)
	Reuteran (27)	glucose	<i>Lactobacillus reuteri</i>

¹ The dietary fibre classes are adapted from Asp (28).

If not indicated, the main constituents and sources are cited from Daniel, et al. (29).

The classification of NSPs has similar challenges to that of dietary fibres, because the majority of dietary fibres are NSPs. The diversity in the chemical structures of NSPs leads to diverse physical properties and different physiological effects. NSPs are often categorised based on solubility in water (30). However, physical properties of NSPs can change depending on many factors, including how the NSPs are extracted (31). The classification based on chemical properties, however, is not always representative for the physiological activities (9).

1.4. Analysis of dietary fibres

With the inclusion of resistant starch and the non-digestible oligosaccharides in dietary fibre category, the analytical method for total dietary fibre was also adapted. The last adaptation of the method includes oligosaccharides as well as resistant starch and non-starch polysaccharides as dietary fibre (32), and this method has been adopted as an official method (AOAC Method 2009.01 (33) and 2011.25 (34)).

In the integrated total dietary fibre assay procedure (32), the polysaccharides are measured gravimetrically and the low molecular weight (LMW) dietary fibres are measured using HPLC. In this method, dietary fibres are measured as a group of compounds, and there is no elucidation of the components or the constituent monosaccharide compositions of the fibres. Also, lignin, which is closely associated with dietary fibres in plant cell wall material, is included in the gravimetric analysis (32). Although the results of the integrated assay comply with the definitions of dietary fibres, they do not give detailed information about the composition of the dietary fibres.

Detailed compositions of dietary fibres can be obtained by measuring the main components of dietary fibres separately. Resistant starch can be measured by another official method (AOAC method 2002.02) (35). The principle of the analysis was based on removal of starch that can be digested by pancreatic α -amylase and amyloglucosidase, followed by enzymatic digestion of the remaining starch and analysis of the glucose from the resistant starch. The constituent monosaccharide composition of the NSPs can be analysed using the method previously developed for neutral monosaccharides (36), coupled with uronic acid analysis (37). The principle of this method is removal of the starch from the sample, followed by hydrolysis of the remaining NSPs and analysis of the monosaccharides

released by the hydrolysis. Alternatively, if the dietary fibres are soluble in water and different uronic acids are present, the method involving methanolysis combined with hydrolysis using trifluoroacetic acid (TFA), followed by High Performance Anion Exchange Chromatography (HPAEC) (38) can be used. For the LMW dietary fibres or oligosaccharides, if the constituent monosaccharide composition is needed, the approach used in the integrated total dietary fibre assay for LMW soluble dietary fibre can be extended by hydrolysis of the oligosaccharides followed by analysis of the resulting monosaccharides. For this purpose, the analysis method for soluble carbohydrates as mentioned above (38) can be used. In addition, there are methods available for analysing specific oligosaccharides such as FOS (39, 40) and GOS (41).

1.5. Analysis of physiological effects of dietary fibres

1.5.1. *In vivo* studies

Experiments involving diet intervention studies of human subjects are irreplaceable for the research about dietary fibres. Nevertheless, research using human subjects is highly restricted because of ethical reasons. Consequently, in order to investigate the mechanisms behind the effects of dietary fibres, animal models are often used.

Rodents, such as mice and rats, are often used as animal models for humans. Rodents have the advantage that they are small and have a rapid growth and reproduction cycle. It is also possible to modify the diet of rodents to contain only a single dietary fibre. Nevertheless, the digestive system of rodents, especially the colon, has been shown to be very different from human (42). Although more costly in time and resources, pigs are regarded as more representative than rodents for studies involving the large intestine, because their large intestine is more similar to humans (42, 43). Another advantage of using pigs as model animals is that pigs can also be equipped with cannulae. Samples from cannulated animals are valuable because samples can be taken from the same animal that received different diets at different periods. Moreover, the animals can be sacrificed to obtain samples that are rarely possible to be obtained from humans, such as tissue samples or digesta from different parts of the GIT. In addition, the results of dietary fibre research in pigs as models for human can also be applied in pig farming practices, and vice versa.

In many studies, the digesta obtained from animals fed with diets containing dietary fibres were measured for contents of short chain fatty acids as fibre fermentation products (44, 45). The digesta are also often analysed for microbiota composition (45-47). If an indigestible marker, such as TiO_2 or Cr_2O_3 , is added into the diets, the apparent digestibility, which is a measure of how much of the consumed dietary fibres are utilised by the microbiota in the GIT, can also be estimated (48-50). Results of dietary fibre digestibility estimations depend on the analysis method used to measure the dietary fibres in the samples. If gravimetric methods, which include lignin, are used, the digestibility result will also include the digestibility of lignin. More detailed results can be obtained if the dietary fibres are analysed for their constituent monosaccharide composition, and/or by analysis methods capable of analysing specific dietary fibres, such as cellulose and mixed-linkage β -glucans (48, 49). Hence, the degradation of individual dietary fibres in the GIT can be monitored.

For research on the degradation of dietary fibres in the GIT, samples from different parts of the colon are invaluable. Using such samples, fermentation of certain dietary fibres along the colon can be monitored. For example, it was revealed that mixed-linkage β -glucans are extensively utilised by the microbiota in the caecum (51). In another study, it was concluded that arabinoxylans from different parts of the rye kernel are degraded by the microbiota at different rates along the large intestine (52). Without taking samples from different parts of the large intestine, drawing these conclusions would not have been possible.

Despite the many analyses done on the digesta from model animals, the degradation products of the dietary fibres were not much studied. During their utilisation by the microbiota, dietary fibres are degraded into lower molecular weight polysaccharides, oligosaccharides or monosaccharides. This was indicated by the presence of polysaccharide degrading enzymes and glycosidases in the fermentation medium after *in vitro* fermentation of various dietary fibres by human faecal microbiota (53). These degradation products may provide more information about the mechanisms of dietary fibre degradation in the GIT than the fermentation end products. However, dietary fibre degradation products have been monitored only in a few studies, and these studies were focused on the upper GIT (54, 55).

1.5.2. *In vitro* studies

In vitro methods have often been used to predict how certain dietary fibres may behave in the body. *In vitro* experiments have the advantage that individual dietary fibres can be tested in pure form without the interference of other compounds. *In vitro* studies performed on dietary fibres include characterisation of the physical properties of dietary fibres and *in vitro* fermentation of dietary fibres. Also, *in vitro* methods have been used to study interactions between dietary fibres, their degradation products, and mammalian cells, such as immune cells and endothelial cells (56).

Characterisation of the physical properties of dietary fibres is mainly related to the hydration properties, including solubility, swelling capacity and water binding capacity. Different methods for measuring water binding capacity have been reviewed (57). Hydration properties of dietary fibres depend on the chemical characteristics and on the particle size of the fibre (58). The mechanisms involved in water binding by dietary fibres have been described in detail elsewhere (59).

In vitro fermentation has been used extensively to study the fermentation of different nutrients including dietary fibres by intestinal microbiota. *In vitro* fermentation has been performed in different ways, from the simple batch system (60) to the continuous system composed of three fermentors set at different pH to simulate the pH changes along the large intestine (61). During *in vitro* fermentation, the fermentation products of different dietary fibres and the substrate utilisation by the microbiota can be monitored (60, 62).

In an attempt to simulate the human digestive system as much as possible, sophisticated models are developed. These models incorporate peristaltic movements, removal of small molecules and the addition of simulated digestive liquids. An example of such a model is the TNO intestinal model (TIM-1), which can simulate the stomach and the small intestine (63). Another model was also developed to simulate the large intestine (64). These models can simulate the *in vivo* conditions better than the simple ones, but they are more expensive and have low throughput (65). Hence, the simple models are still valuable when a lot of samples need to be analysed.

Similar to the digesta from the *in vivo* studies, samples from *in vitro* fermentation are often analysed for gasses, SCFA content, substrate disappearance, and microbiota composition (65-70). However, the fate of the dietary fibre itself is seldom monitored during

fermentation. In the few studies that did monitor the degradation of dietary fibres during *in vitro* fermentation, it was concluded that xylo-oligosaccharides (XOS) that are acetylated or substituted with 4-O-methyl glucuronic acid were more difficult to ferment by human faecal microbiota than unsubstituted XOS or arabinose-substituted XOS (71). In another study, it was shown that unsaturated pectin oligosaccharides were formed during *in vitro* fermentation of pectin by human faecal microbiota (72). Such conclusions are important in explaining the mechanisms of dietary fibre degradation by the intestinal microbiota. They may lead to an understanding on the types of oligosaccharides that can be expected to be present in the large intestine upon consumption of a dietary fibre.

The oligosaccharides as degradation products of dietary fibres in the large intestine can be of special interest because oligosaccharides have been shown to have direct interaction with immune and endothelial cells (56). Using *in vitro* techniques, it could be revealed which oligosaccharides can interact with certain cells, and which part of the oligosaccharide is important for its recognition by the cell. As an example, it was shown that immune cells tend to react to oligosaccharides with helical conformation (73).

1.6. Health effects of dietary fibres

Dietary fibres have been claimed to have a role in regulating body weight (74), alleviate diabetes (75), prevent cardiovascular diseases (76), maintain colon health (77), and even prevent various cancers (78-80). In the description that follows, the mechanisms that are known about how dietary fibres can influence health at different sites in the gastrointestinal tract (GIT) are described.

1.6.1. Dietary fibre in the upper gastrointestinal tract

The presence of dietary fibres in food potentially reduce energy intake. This effect was mentioned as 'intrinsic effects' in the review of Slavin (1). In food, dietary fibres provide bulk volume with less energy compared to other nutrients, thus reducing the energy density of food (81).

The effects of dietary fibres after consumption start in the mouth. Foods that are rich in dietary fibre are often regarded as less palatable (81), and they often require a lot of chewing. Foods with low palatability (82) and chewy foods (83, 84) may reduce food

intake. Further along the upper gastrointestinal tract, the effects of dietary fibres in the stomach and in the small intestine are often assumed to be related to their physical characteristics, mainly viscosity and gelling ability (58). It was shown that a high viscosity meal may delay gastric emptying (85, 86) and induce satiety hormones (86). Gastric emptying is one of the many factors that have a role in satiation and regulation of food intake (87, 88).

In the small intestine, increased viscosity of the digesta has been related to a decreased rate of nutrient absorption and reduction of glycaemic response (89). Besides having a role in the regulation of food intake (87, 88), the reduction of the glycaemic index in food can prevent the onset of diabetes type 2 (90). The effects of viscous dietary fibres on glycaemic response, blood lipids, intestinal enzymatic activity and nutrient digestibility have been documented in detail (91).

The effects of dietary fibres described above are due to the increased viscosity of gastrointestinal liquid. Dietary fibres without viscous or gelling properties, therefore, are often found not having much effect in the upper GI tract. It was shown, however, that water insoluble dietary fibres with high water holding capacity can reduce the free water content and increase the viscosity of the digesta (92). Most oligosaccharides are soluble in water, but have a low viscosity. These oligosaccharides may have a different role in the upper GIT, because some dietary fibres, including oligosaccharides, are able to interact with immune cells (73, 93).

1.6.2. Dietary fibres in the large intestine

After passing the upper GIT, the dietary fibres enter the large intestine. In the large intestine, there is a large community of microbiota that utilises the dietary fibres as carbon sources for their growth. The colon microbiota, which may comprise of thousands of species (94), can compose more than 40 % of the total solids in human faeces (95). It was proposed that the fermentation of dietary fibres by the microbiota is one of the main mechanism by which dietary fibres can influence health (96).

Dietary fibres that can be fermented by the gut microbiota potentially modify the composition of the microbiota. Some dietary fibres are shown to be able to increase the diversity of the microbiota. In addition, especially fructo-oligosaccharides and galacto-

oligosaccharides are reported to selectively stimulate the growth of *Bifidobacteria* and *Lactobacilli* (12), which are considered as beneficial for health (97). The term 'prebiotic' is used for dietary fibres which are able to selectively promote the growth of beneficial bacteria in the large intestine (97).

The end products of dietary fibre fermentation in the large intestine are gasses, such as hydrogen or methane, next to short chain fatty acids (SCFA). The SCFA are mainly acetic acid, propionic acid and butyric acid. It has been shown that the amounts of these SCFA in human faeces increased when fermentable dietary fibres are consumed (96). The SCFA production was highest in the caecum (98). An increased amount of SCFA in the digesta is associated with a lower pH of the digesta, which inhibits the growth of pathogenic bacteria (99). More than 95 % of the SCFA produced by the microbiota was absorbed by the intestinal cells of the host (100). Acetic acid, propionic acid, and butyric acid have been shown to influence various processes in the body, including satiety, cholesterol metabolism, carcinogenesis, and insulin sensitivity (94).

Dietary fibres and their degradation products that are not well fermented by the microbiota in the large intestine are beneficial in maintaining healthy stool output. It has been reported that one of the factors which determine the effects of dietary fibres to the stool output is the water holding capacity of the fibre (101). The unfermented dietary fibres and the water bound by the fibres increase the volume of digesta in the large intestine. With an increased digesta volume, the toxic substances that may be present in the digesta are diluted. Hence, the interactions between toxic substances and intestinal cells may be reduced (102). Increased digesta volume may also result in decreased transit time. Shorter transit time may reduce water absorption from the digesta and maintain the stool consistency within the healthy range.

1.7. Thesis outline

As was described above, the effects of dietary fibres in the large intestine is often related to the fermentation of the fibre and the fermentation products, especially SCFA. The degradation of the dietary fibres itself was seldom monitored. Hence, the intermediate products of dietary fibre degradation, which can be oligosaccharides, are usually ignored.

The aim of this thesis was to monitor the degradation of dietary fibres along the gastrointestinal system in *in vitro* simulation and *in vivo* experiments.

First, the behaviour of three dietary fibres with different physicochemical properties in the upper GIT and their possible roles in satiation were studied (**Chapter 2**). *In vitro* experimental results were discussed in relation to the human satiation responses. Dietary fibres will eventually end up in the large intestine and being fermented by the microbiota. Hence, the fermentation of dietary fibres with a broad variation in their chemical properties was studied *in vitro* (**Chapter 3**). During fermentation, fermentation products and dietary fibre degradation products were monitored. A comparison was also made between fermentation by faecal microbiota from humans and that from pigs.

Pigs were used in subsequent experiments as models for human (**Chapters 4-6**). In the *in vivo* studies, resistant starch was used as the main added fibre because it has been found to be a satiating fibre (103). In **Chapter 4**, the effects of resistant starch on the degradation of NSP from wheat and barley are described. In this study, polysaccharide degrading enzyme activity in the digesta along the large intestine was used for estimating the extent of fibre degradation. **Chapter 5** focuses on the degradation of alginate, which is known as a slowly fermentable fibre, in the large intestine of pigs. The alginate was added into diets without or with resistant starch. Alginate and alginate degradation products, which resisted catabolism by the microbiota and were secreted in the faeces, were characterized. The method developed for analysing the alginate oligosaccharides and the animal to animal variation in alginate degradation are discussed in **Chapter 6**. The results gained from *in vitro* and *in vivo* studies, as well as the consequences of the insights gained in this thesis for dietary fibre research, as well as for the production and consumption of dietary fibres are discussed in the last chapter (**Chapter 7**).

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Chapter 2

The effects of bulking, viscous and gel-forming dietary fibres on satiation

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Abstract

The objective was to determine the effects of dietary fibre with bulking, viscous and gel-forming properties on satiation, and to identify the underlying mechanisms. We conducted a randomised crossover study with 121 men and women. Subjects were healthy, non-restrained eaters, aged 18–50 years and with normal BMI (18.5–25 kg/m²). Test products were cookies containing either: no added fibre (control), cellulose (bulking, 5 g/100 g), guar gum (viscous, 1.25 g/100 g and 2.5 g/100 g) or alginate (gel-forming, 2.5 g/100 g and 5 g/100 g). Physico-chemical properties of the test products were confirmed in simulated upper gastrointestinal conditions. In a cinema setting, *ad libitum* intake of the test products was measured concurrently with oral exposure time per cookie by video recording. In a separate study with ten subjects, 4 h gastric emptying rate of a fixed amount of test products was assessed by ¹³C breath tests. *Ad libitum* energy intake was 22 % lower for the product with 5 g/100 g alginate (3.1 ±1.6 MJ) compared to control (4.0 ±2.2 MJ, P <0.001). Intake of the other four products did not differ from control. Oral exposure time for the product with 5 g/100 g alginate (2.3 ±1.9 min) was 48 % longer than for control (1.6 ±0.9 min, P =0.01). Gastric emptying of the 5 g/100 g alginate product was faster compared to control (P <0.05). We concluded that the addition of 5 g/100 g alginate (i.e. gel-forming fibre) to a low fibre cookie results in earlier satiation. This effect might be due to an increased oral exposure time.

2.1. Introduction

The consumption of dietary fibre has been associated with increased satiety and reduced energy intake (1-5). Satiety and satiation are part of a complex system of appetite control, including cognitive factors, sensory sensations and post-ingestive feedback mechanisms (6). Satiety is defined as the inhibition of appetite and occurs as a consequence of eating. Satiation is defined as the satisfaction of appetite that develops during the course of a meal, and results in meal termination. Numerous studies have been carried out to clarify the effects of dietary fibre on satiety (4, 5, 7). Studies on the effects of fibre on satiation are, however, limited and show inconsistent results. For example, Grimes and Gordon (8) found that the satiating capacity of wholemeal bread was higher than that for white bread. Opposing to this, Burley, et al. (9) did not find differences in *ad libitum* intake between a meal containing a meat replacer with chitin and insoluble β -glucan and a similar low fibre meal. Odunsi, et al. (10) also did not find differences in *ad libitum* intake after ingestion of capsules with cellulose and alginate compared to placebo capsules.

Dietary fibre is a term that reflects a heterogeneous group of compounds that differ in their chemical structure and physico-chemical properties. Dietary fibres may affect satiation via diverse related mechanisms (7, 11). First, the metabolisable energy content of fibre is less than that for other nutrients (12) and, as meal intake volume is relatively constant (13), the inclusion of fibre in foods decreases total energy intake. Second, adding fibre to a meal can increase chewing activity or oral exposure time to foods, which may result in earlier satiation (14-16). Third, the addition of fibre can increase viscosity and water-holding capacity of digesta and induce formation of gels in the stomach (11, 17). These properties can slow down gastric emptying and concurrently increase stomach distension. Stomach distension, or fullness, is seen as a causal factor in the chain of events leading to satiation (18, 19). In response to the mechanical and physico-chemical properties of the ingested foods, a series of neural and humoral signals develop from the gut, which can result in satiation (20).

The aim of the present research was to determine the effects of three distinctive dietary fibres with different physico-chemical properties on satiation. Hence, we selected cellulose, a bulking fibre; guar gum, a viscous fibre; and alginate, a gel-forming fibre, and added the selected fibres to test products. Two dosages of guar gum and alginate were included to be

able to study effects of high fibre, but less palatable products. Physico-chemical properties of the test products were characterised in simulated upper gastrointestinal conditions. Satiation was determined by measuring *ad libitum* intake of the test products in a real-life setting. Furthermore, oral exposure time and gastric emptying rate were measured.

2.2. Subjects and methods

Two short-term intervention studies were conducted. Satiation and oral exposure time were determined in study one, and gastric emptying rate was assessed in study two. In both studies, the subjects participated in six test sessions with six different test products.

2.2.1. Subjects

For both studies, men and women, aged 18–50 years, were recruited in Wageningen and Ede, The Netherlands. Subjects had to have a normal BMI (18.5–25.0 kg/m²), and had to be healthy. Subjects were excluded if they were restrained eaters according to the Dutch Eating Behaviour Questionnaire (DEBQ) (score: men >2.89; women >3.39) (21). They were also excluded if they used an energy-restricted diet or lost or gained more than 5 kg body weight during the last 2 months, if they had a lack of appetite, had diabetes, gastrointestinal problems or were hypersensitive for any ingredient in the test products. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Medical Ethics Committee of Wageningen University (registration no. NL 26703.081.09). Written informed consent was obtained from all subjects. The study was registered in the National Institutes of Health clinical trial database (ClinicalTrials.gov no. NCT00904124).

Out of the 124 subjects in study one, three dropped out due to reasons unrelated to the intervention. We included 121 subjects in data analysis, of which 112 participated in six sessions, seven in five sessions and two in four sessions. The missed sessions were due to illness or problems with planning. The study population for study one consisted of forty-five men and seventy-six women, aged 25 ±7 years, with a BMI of 22.0 ±1.9 kg/m² and a DEBQ score of 2.1 ±0.6. The number of women in the menstrual phase did not differ ($P=0.79$) between treatments.

A total of ten subjects, six men and four women, participated in study two. All subjects were included in data analysis, of which nine participated in six sessions and one in five sessions. The missed session was due to problems with planning. Mean age of the participants was 21 ± 3 years, mean BMI 21.8 ± 1.9 kg/m² and mean DEBQ score 1.8 ± 0.7 .

2.2.2. Test products

The six test products were one-bite-sized (6.8 ± 0.3 g) chocolate cookies. The basic recipe of the cookies contained 36 % white flour, 27 % butter, 18 % sugar, 14 % chocolate chips, 4 % egg, 2 % cacao powder and 0.1 % salt. Flour was exchanged for dietary fibre. Cellulose (Vitacel L 00, Rettenmaier & Söhne) was given in a dose of 5 %; guar gum (Viscogum™ MP 41 230, Cargill; molecular weight 60–1000 kDa) in doses of 1.25 and 2.5 % and alginate (Protanal LF 5/60, FMC BioPolymer; molecular weight 17–710 kDa; guluronic acid:mannuronic acid ratio of 1.9) in doses of 2.5 and 5 %. A professional bakery manufactured the cookies freshly on each test day.

Duplicate portions of the products were collected on each test day and stored at -20°C pending measurements for macronutrients and physico-chemical properties. Before measurements, a homogenised mixture of cookies was ground until it passed a 2 mm sieve. Protein, total fat, total dietary fibre, moisture and ash were measured according to methods previously described (22). Available carbohydrate was estimated by subtracting moisture, ash, protein, fat and fibre from total weight. Atwater factors were used to calculate available energy: fat 37 kJ/g and protein and carbohydrate 17 kJ/g. For fibre, 0 kJ/g was used because of uncertainty about the availability of energy (12). This may have underestimated the available energy content. Macronutrient composition is shown in Table 2–1.

Physico-chemical properties were measured only for the high-dose products and the control. These properties included viscosity and water-holding capacity using three conditions to simulate the mouth, stomach and small intestine. Measurements were performed according to methods described by Turnbull, et al. (23), with modifications for the amount of samples and types of reagents. Reagents used included α -amylase from porcine pancreas (1.16.312.0001, Merck), pepsin from porcine gastric mucosa (P6887, Sigma-Aldrich), pancreatin from porcine pancreas (P1625, Sigma-Aldrich) and bile extract (B8631, Sigma-Aldrich). The amount of sample was increased by 4-fold, to compensate for

lower fibre levels. Furthermore, the volume for each simulation was set to 30 ml, and amounts of sample and reagents were adjusted comparatively. In addition, amounts of enzymes were adjusted to obtain similar activity. Bile was increased by 4-fold to ensure good emulsification of fat. After each simulation, samples were centrifuged at 4250 ×g for 20 min. The supernatant was decanted and used for viscosity measurements. The tube with the remaining pellet was inverted to remove excess water. The pellet that contained insoluble material was weighed and the DM was measured. Water-holding capacity was expressed as the amount of water held after centrifugation by the insoluble material from 1 g of cookie.

Table 2–1. Available energy and macronutrient composition of the test products (per 100 g).

Component	Control		Cellulose 5 %		Guar gum 1.25 %		Guar gum 2.50 %		Alginate 2.50 %		Alginate 5 %	
	g	En%	g	En%	g	En%	g	En%	g	En%	g	En%
Fat	33.3	55	32.2	57	33.3	56	33.2	57	33.2	56	33.2	58
Protein	6.4	5	5.9	5	6.3	5	6.2	5	6.5	5	5.8	5
Available carbohydrate	53.1	40	46.7	38	51.0	39	49.3	39	49.6	39	46.8	38
Dietary fibre	3.6		10.6		5.6		6.9		6		9	
Available energy (kJ) ¹	2241		2087		2204		2171		2180		2122	

En%: percentage of energy, as derived from the specific nutrient compared to the total calculated energy content of the test product.

¹ Available energy was calculated based on chemical analysis of the macronutrient composition. Energy conversion factors used: fat 37 kJ/g, protein and carbohydrate 17 kJ/g. Energy content of fibre was set at 0 kJ/g.

The viscosity of the supernatant was measured at 37 °C, using a rheometer (MCR 501, Anton Paar) with double gap geometry. A shear sweep was performed at 1–1000 /s in logarithmic scale during 5 min. The data obtained at shear rate of 100 /s were used to compare between samples.

2.2.3. Experimental procedure: study one

Ad libitum intake was measured in a randomised single-blind cross-over study with six test sessions, separated by at least 2 d. *Ad libitum* intake was calculated from the weight of the test products before and after consumption. Products were weighed in duplicate on a digital

scale, with a precision of 0.1 g. Subjects were not aware that the primary outcome was *ad libitum* intake, as this could have affected the outcome of the study.

The study was performed in a cinema (Cinemec) to create a real-life setting aimed to distract subjects from visual and weight cues (24). During each test session, subjects watched a movie in the genres romance or comedy. On each test day, the subjects arrived at 18.00 hours. At 18.45 hours, they were seated in the theatre. Just before entering the theatre, 400 g of test product was served in a white carton box and a bottle with 500 ml water was provided. The subjects were instructed to eat as little or as much of the test product as they wanted until they felt comfortably full. The movie was divided in two parts of 45 min, with a 15 min break. During the break, subjects left the theatre and handed in the box with test product. At the restart, they received a new box with 400 g of test product. The participants were instructed to finish the bottle of water before the end of the movie.

Before and after *ad libitum* intake, subjects rated five appetite questions on 100 mm visual analogue scales. Scales were anchored from ‘not at all’ to ‘very much’ and included feelings of hunger, fullness, desire to eat, prospective food consumption and thirst. Before *ad libitum* intake, the participants were also asked to rate palatability, expected satiation and sensory attributes (sweetness, bitterness, chocolate taste, freshness, dryness, stickiness and difficulty to swallow) of the test product on 100 mm visual analogue scales.

To standardise the individual state of satiety, subjects were instructed to eat the same breakfast and lunch at all six test days and to record this in a diary. Individual state of satiety was further standardised by consuming a preload at 18.00 hours. The preload provided approximately 18 % of the daily energy requirements. This was chosen to correspond to half the energy content of a normal Dutch dinner (25). Individual energy requirements were calculated by the Schofield equation (26), and subjects were divided into one of three preload groups. Group one (estimated energy need ≤ 10 MJ, n 63) received 0.5 pizza, group two (10–14 MJ, n 56) received 0.75 pizza and group three (≥ 14 MJ, n 2) received 1.0 pizza.

2.2.4. Oral exposure time

Oral exposure time of the test products was measured by means of video recording a random subgroup of eleven men and twenty-five women. To record eating time, five video

cameras were used (Sony Handycam DCR-HC51/DCR-SR55E; Sony). These were set at night shot mode and supported by two separate IR lights. Video analysis on oral exposure time over the first 45 min of the movie was done through The Observer[®]XT9 (Noldus). Oral exposure time was measured in seconds and defined as time spent on chewing, swallowing, cleaning the mouth and teeth with tongue or fingers. Breaks were considered as not eating. Two researchers coded the video recordings. Reliability analysis was carried out regularly, which resulted in an inter-observer agreement of $\kappa = 0.75$ ($P < 0.01$). Due to varying reasons (e.g. view blocked, poor quality of light) videos of twenty-one to twenty-seven subjects per test product were suitable for quantifying oral exposure time.

2.2.5. Experimental procedure: study two

In a second randomised single-blind crossover trial, gastric emptying rate and appetite sensations were measured in six test sessions, separated by at least 7 d. Subjects consumed a fixed amount of the test products, which corresponded to approximately 20 % of daily energy requirements (25). This resulted in dosages varying from 80 to 100 g. Each portion was supplemented with 87.4 mg [$1\text{-}^{13}\text{C}$] octanoic acid (Campro Scientific GmbH). Breath samples were collected by breathing into a 10 ml Exetainer tube (Labco) via a drinking straw and then closing the tube with a cap. Samples were stored at room temperature and were analysed for ^{13}C enrichment in CO_2 on a Finnigan Delta C continuous-flow isotope ratio mass spectrometer (Finnigan MAT).

Subjects arrived at our research centre between 07.30 and 08.00 hours after a 10 h overnight fast. They were asked to consume a low fibre meal on evenings before test sessions. In addition, they should avoid unusual vigorous physical activity and consuming products naturally enriched in ^{13}C (maize, millet, sorghum and cane sugar). Before ingestion of the test product, within 10 min together with 300 ml water, two baseline breath samples were taken. Subsequent breath samples were taken after exactly 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210 and 240 min. Appetite sensations were rated on 100 mm visual analogue scales, as described for study one, and measured at baseline and after 30, 60, 90, 120, 150, 180, 210 and 240 min. Subjects were seated at a desk and allowed to do light desk work during the session.

2.2.6. Statistical analysis

Data are presented as means and standard deviations. Statistical analyses were performed with SAS (version 9.2; SAS Institute, Inc.). Significance was set at $P < 0.05$. One-way ANOVA was used to analyse differences between physico-chemical properties of the cookies. For study one, treatment effects on sensory ratings, palatability ratings, *ad libitum* intakes and eating time were analysed by means of a mixed-model ANOVA (proc mixed). Treatment, day and treatment \times day interaction (=order) were included as fixed factors and subject was included as a random factor. For dose–response effects, orthogonal contrasts among control, low- and high-dose fibres were calculated. If the treatment effect was statistically significant, Dunnett's procedure was used to compare the fibre treatments with the control treatment, to control for multiple testing. The appetite ratings were analysed according to a similar procedure, with the addition of time (before and after *ad libitum* intake) and treatment \times time as fixed factors in the model. Additionally, to control for differences in appetite ratings at baseline, baseline values were added to the model as a covariate. For study two, treatment effects were analysed according to a similar procedure, after calculation of total area under the curve (AUC) for appetite ratings and gastric emptying rate (proc expand). Time-to-peak data were not normally distributed and were therefore log-transformed for analysis and presented as back-transformed geometric means (95 %CI). Pearson's partial correlation coefficient, controlled for subject, was calculated to assess relations among sensory properties, palatability and *ad libitum* intake for the treatments separately and together.

2.3. Results

2.3.1. Physico-chemical properties

Physico-chemical properties of the test products in simulated upper gastrointestinal conditions are presented in Table 2–2. Under mouth-like conditions, high-dose guar gum increased viscosity up to 24-fold compared to control ($P < 0.001$). The increased viscosity for high-dose guar gum persisted under simulated conditions for stomach and small intestine ($P < 0.001$). High-dose alginate increased water-holding capacity up to 3-fold in the stomach-like conditions compared to control ($P < 0.001$).

Table 2–2. Viscosity and water-holding capacity of the test products in simulated upper gastrointestinal conditions.

Properties	Control		Cellulose 5%		Guar gum 2.5%		Alginate 5%		p ¹
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Viscosity (mPa.s) ²									
Mouth	1.4	0.2	1.3	0.3	34.5 ***	9.4	5.9	0.7	<0.001
Stomach	1.2	0.3	1	0.1	8.4 ***	1.8	1.7	0.4	<0.001
Small intestine	2.5	1.3	3.5	1.1	5.6 ***	0.9	4.1 *	0.8	<0.001
Water holding capacity (g water/g cookie) ³									
Mouth	0.41	0.02	0.47	0.06	0.7 ***	0.02	0.37	0.01	<0.001
Stomach	0.47	0.06	0.53	0.06	0.48	0.02	1.51 ***	0.12	<0.001
Small intestine	0.28	0.06	0.46	0.07	0.37	0.06	0.33	0.12	0.052

Mean values were significantly different from control: * P <0.05, *** P <0.001.

¹ P-value from one-way ANOVA, subsequently all fibre treatments were compared to control with Dunnett's procedure.

² Viscosity in mPa.s at shear rate 100 /s; mean of six measurements.

³ The amount of water held by the insoluble material from 1 g of cookie; mean of four measurements.

2.3.2. Study one

2.3.2.1. Palatability and sensory ratings of test products

Mean palatability and sensory ratings of the test products are given in Table 2–3. Products with cellulose (P <0.001), high-dose guar gum (P =0.001) and high-dose alginate (P =0.023) were rated lower on palatability than control. Expected satiation was rated similar for all test products compared to control. All fibre-enriched products changed in texture ratings compared to the control product. The products with cellulose, high-dose guar gum and both dosages of alginate were rated to be more sticky (P <0.001) than control.

Table 2-3. Palatability ratings, expected satiation and analytical attributes¹ by test products, before ad libitum intake.

Attribute	Control		Cellulose		Guar gum		Guar gum		Guar gum		Alginate		Alginate		p ²
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Palatability	66	17	51 ^{***}	21	62	17	59 ^{**}	17	63	17	61 [*]	18	61 [*]	18	<0.001
Expected satiation	52	19	53	18	49	18	51	17	52	18	55	16	55	16	0.022
Sweetness	58	18	50 ^{***}	20	55	19	48 ^{***}	21	52 [*]	19	55	18	55	18	<0.001
Bitterness	30	20	29	21	32	21	31	21	30	21	28	20	28	20	0.082
Chocolate taste	64	17	57 ^{**}	18	58 [*]	19	53 ^{***}	20	60	17	59 [*]	19	59 [*]	19	<0.001
Freshness	68	20	41 ^{***}	22	64	19	56 ^{***}	20	64	20	63 [*]	19	63 [*]	19	<0.001
Dryness	45	22	58 ^{***}	23	50	21	52	22	50	23	53 ^{**}	23	53 ^{**}	23	<0.001
Stickiness	40	23	49 ^{**}	23	47	24	55 ^{***}	24	57 ^{***}	23	71 ^{***}	16	71 ^{***}	16	<0.001
Difficulty to swallow	31	21	42 ^{***}	26	37	22	41 ^{***}	24	42 ^{***}	25	51 ^{***}	25	51 ^{***}	25	<0.001

Mean values were significantly different from control: * P < 0.05, ** P < 0.01, *** P < 0.001.

¹ Values were measured on a 100 mm visual analogue scale anchored from 'not at all' to 'very much' (0 to 100). Measured in 121 subjects.

² P-value from mixed-model ANOVA, subsequently all fibre treatments were compared to control with Dunnett's procedure.



2.3.2.2. Appetite ratings

After *ad libitum* intake, ratings for hunger, desire to eat and prospective consumption decreased ($P < 0.001$) and ratings for fullness increased ($P < 0.001$) for all test products compared to before *ad libitum* intake. The change in ratings compared to baseline did not differ between test products (data not shown).

2.3.2.3. *Ad libitum* intake

Figure 2–1 shows the total *ad libitum* intake of the test products. Before the break, at 45 min, *ad libitum* intake represented 67–70 % of total intake for all test products. Intake of the products containing cellulose, both dosages of guar gum and the low-dose alginate did not change compared to the control product, regardless of the dimension used (i.e. g or MJ). Compared to the control product, high-dose alginate reduced *ad libitum* intake in grams by 17 % ($P < 0.001$), which corresponded to a reduction in MJ of 22 % ($P < 0.001$). In addition, a dose–response effect of alginate was found; increasing fibre dose reduced *ad libitum* intake ($P < 0.05$).

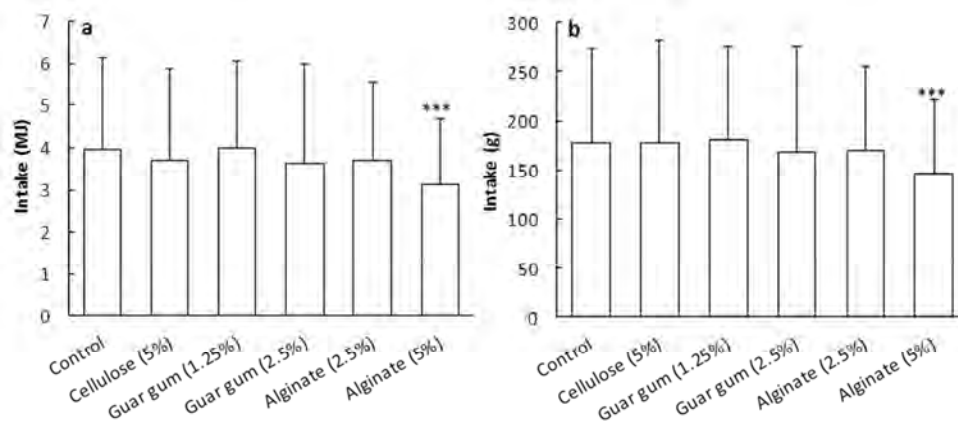


Figure 2–1. *Ad libitum* intake of the test products in (a) MJ (sd) (n 121) and (b) g (sd) (n 121). Analysis with mixed-model ANOVA resulted in $P < 0.001$, subsequently all fibre treatments were compared to control with Dunnett's procedure. Orthogonal contrasts among control, low- and high-dose guar gum and alginate showed a dose–response effect of alginate ($P < 0.05$). ***Values were significantly different from control ($P < 0.001$).

2.3.2.4. Low resolution version High resolution version

Palatability scores were positively correlated with *ad libitum* intake ($r 0.17$; $P < 0.001$). For the individual products, this correlation was only found for test products containing cellulose ($r 0.18$; $P = 0.045$), low-dose guar gum ($r 0.40$; $P < 0.001$) and high-dose guar gum

(r 0.19; P =0.041). Scores for stickiness were inversely correlated with *ad libitum* intake (r -0.10; P =0.008), but this was not found for the individual test products. Adjusting the results of *ad libitum* intake for palatability and stickiness of the test products, by including these variables as covariates in the model did not change the findings.

2.3.2.5. Oral exposure time

In the subgroup for video analysis (n 36), *ad libitum* intake of test products did not differ from the intake in the complete group. Although there was an effect of treatment on total oral exposure time (P =0.045), this effect could not be localised to specific test products compared to control (Table 2–4). Oral exposure time per cookie was only longer for the high-dose alginate, compared to control (P =0.01).

2.3.3. Study two

Table 2–5 shows the AUC and time to peak for gastric emptying. Compared to control, AUC for gastric emptying was larger after consumption of the products with cellulose (P =0.048), low-dose alginate (P =0.027) and high-dose alginate (P =0.004). Additionally, time to reach the peak percentage dose recovery of ^{13}C per h was 27 % shorter for high-dose alginate compared to control (P =0.03). AUC for 4 h ratings of hunger, fullness, desire to eat and prospective consumption did not differ between the test products and control (data not shown).

Table 2-4. Total oral exposure time and oral exposure time¹ of the test products measured by video observation.

	Control		Cellulose 5 %		Guar gum 1.25 %		Guar gum 2.5 %		Guar gum 2.5 %		Alginate 2.5 %		Alginate 5 %		p ²
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Total oral exposure time (min) ¹	20.2	8.9	20.2	10.6	24.9	11.7	24.1	14.0	22.3	9.5	22.9	12.5	22.9	12.5	0.045
Oral exposure time per cookie (min)	1.6	0.9	1.7	1.5	1.7	1.3	2	1.6	1.7	0.8	2.3	**	1.9	0.043	
Oral exposure time/MJ (min)	9.4	4.9	10.7	8.1	10.2	7	11.8	9.1	10.1	4.5	13.1	**	8.3	0.031	

Mean values were significantly different from control: ** P < 0.01.

¹ Total oral exposure time and oral exposure time per cookie are reported in minutes over the first 45 min of a test day. Measured in a subgroup of thirty-six subjects.

² P-value from mixed-model ANOVA, subsequently all fibre treatments were compared to control with Dunnett's procedure.

Table 2-5. Gastric emptying rate by test product expressed as area under the curve (AUC) and time to peak¹.

	Control			Cellulose, 5 %			Guar gum, 1.25 %			Guar gum, 2.5 %			Alginate, 2.5 %			Alginate, 5 %			p ²
	Mean	95 %CI	n	Mean	95 %CI	n	Mean	95 %CI	n	Mean	95 %CI	n	Mean	95 %CI	n	Mean	95 %CI	n	
AUC	1780	1513	2045 *	1769	1918	1650	1864	1579	2126 *	1860	2145 **	1877	2412	2392	2126 *	1860	2145 **	1877	0.007
Time to peak (min)	83	56	64	41	81	59	99	67	66	44	61 *	45	0.006	123	102	110	148	99	83

CI: Confidence intervals.

Mean values were significantly different from control: * P < 0.05, ** P < 0.01.

¹ Gastric emptying was measured as percentage dose recovery of ¹³C per h after ingestion of a fixed amount of test product. Total AUC over 240 min was calculated according to the trapezoid method. An increase in AUC reflects an increased amount of test product that is emptied into the duodenum over 240 min. Data on time to peak were log-transformed for analysis and are presented as back-transformed geometric means (95 %CI).

² P-value from mixed model ANOVA, and subsequently all fibre treatments were compared to control with Dunnett's procedure.

2.4. Discussion

In the present study, we found that cookies supplemented with 5 % alginate (i.e. gel-forming fibre) reduced *ad libitum* intake in energy by 22 %, compared to cookies without added fibre. Addition of guar gum (i.e. viscous fibre) and cellulose (i.e. bulking fibre) did not affect *ad libitum* intake. Cookies with 5 % alginate increased oral exposure time by 48 %, but also increased the rate of gastric emptying. The present study was performed in a real-life setting to distract subjects from visual and weight cues. We included two different dosages of guar gum and alginate to be able to study effects of high fibre, but less palatable products.

Selection of the types of fibre for the present study was based on anticipated working mechanisms of bulking, viscous and gelling fibres on satiation. By definition, all fibres have bulking properties, as inclusion of dietary fibre in food products reduces energy density (12). In the present study, *ad libitum* intake in weight remained unchanged after inclusion of cellulose compared to the control product without added fibre. The change in energy content after inclusion of cellulose was, however, not large enough to lead to significant decreases in energy intake.

In addition to weight or volume of foods, palatability is an important determinant of meal size (27). A very pleasant-tasting meal may result in higher *ad libitum* intake. In the present study, palatability ratings for the high-dose fibre products were lower than that for the control product. However, adjusting for palatability did not explain the difference in *ad libitum* intake between high-dose alginate and control products.

We hypothesised that addition of guar gum would reduce *ad libitum* intake (14, 28) by increasing oral exposure time (16, 29). The measurements of physico-chemical properties confirmed that guar gum was highly viscous in mouth conditions. However, in the satiation study, we showed that guar gum neither reduced *ad libitum* intake nor increased oral exposure time. Although there were texture differences, we speculate that these were not large enough to prolong oral exposure time (30). Previous studies showing effects on oral exposure time used liquid and semi-liquid test products with large differences in texture (14, 28).

While no effect of guar gum was observed, oral exposure time increased after high-dose alginate supplementation, although viscosity in the simulated mouth condition did not differ from control. Alginate forms a gel either at a low pH or in the presence of divalent cations (e.g. Ca^{2+} or Mg^{2+}) (31). We postulate that alginate already started forming a gel in the oral cavity due to the presence of water and divalent cations from saliva (32). This is also in agreement with the sensory ratings, as alginate was rated the most sticky and difficult to swallow.

We further hypothesised that increased viscosity of digesta as well as formation of gels would reduce gastric emptying rate, and as a result reduce *ad libitum* intake (11, 17, 19). The measurements of physico-chemical properties confirmed that guar gum increased viscosity in all three upper gastrointestinal conditions, and that alginate increased water-holding capacity in stomach conditions. In the gastric emptying study, we found, however, that none of the test products reduced gastric emptying rate. Gastric emptying rate even increased for alginate. Previous findings on the effects of viscous fibre (29, 33, 34) and gelling fibre (10, 17) on gastric emptying have also been inconclusive. Despite this, increased viscosity as well as gel formation in digesta generally results in prolonged presence of nutrients in the small intestine, which in turn inhibits the absorption of glucose in blood and affects appetite-regulating peptides (35). This process may have contributed to the reduced intake of high-dose alginate cookies in the present study.

The initial hypotheses on oral exposure time, gastric emptying rate and *ad libitum* intake could not be confirmed. This may be explained by the rate of hydration. When mixed with liquids (e.g. saliva and gastric secretion), viscous and gelling fibres are expected to be hydrated and induce thickening or form a gel. The thickening of a fibre depends not only on factors such as structure, dose and molecular weight, but also on the rate of hydration (35-37). For gelling fibre, factors such as dose, pH, presence of Ca^{2+} and rate of hydration are crucial (31). In the simulation study, the test product was finely ground and the incubation time in mouth, stomach and small-intestinal conditions were relatively long, respectively, 10, 60 and 180 min (23). In real life, oro-gastric transit time may be faster, so fibres may not have been fully hydrated before arriving in the stomach and therefore not behave according to the anticipated working mechanisms.

In the present study, we showed that physico-chemical properties of fibres can affect food intake and satiation-related mechanisms in the upper gastrointestinal tract. Apart from the physico-chemical properties, as determined in simulated conditions, it should be realised that intraluminal conditions in the upper gastrointestinal tract, such as interactions with the digesta matrix, pH, hydration status and passage rate, have an impact on fibre properties and post-meal effects *in vivo*.

It is important to note that fibre properties associated with satiation (i.e. gel-forming in the present study) may not automatically be associated with a reduced energy intake or sustained satiety after repeated exposure. We previously showed that in the short term, viscous fibre increased satiety more than non-viscous fibre, whereas in the longer term, effects on energy intake and body weight were independent of viscosity (7). Other mechanisms related to specific fibre properties, such as secretion of appetite-regulating peptides, inhibited absorption of nutrients from the lumen, enhanced insulin sensitivity and enhanced prebiotic activity, may interplay and affect energy intake or sustained satiety (38, 39).

2.5. Conclusions

Addition of 5 g/100 g alginate (i.e. gel-forming fibre) to a low fibre cookie resulted in earlier satiation in a real-life setting. This effect may be mediated by an increased oral exposure time. Guar gum (i.e. viscous fibre) and cellulose (i.e. bulking fibre) did not affect *ad libitum* intake. Fibre properties can change after interaction with the food matrix and the environment in the upper gastrointestinal tract, and as a result this can change the effect on satiation.

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Chapter 3

In vitro fermentation of 12 dietary fibres by faecal inoculum from pigs and humans

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Abstract

In vitro fermentation of 12 dietary fibres by faecal inocula from pigs and humans were performed. The fibres included homoglucons, mannans, fructans, polyuronides, and complex heteroglycans. Gas production, short chain fatty acid production and fibre degradation products were monitored during fermentation. Human inoculum has more ability to ferment resistant starch and fibres containing uronic acids. In contrast, pig inoculum is able to ferment cellulose, which is hardly fermented by human inoculum. The constituent monosaccharide and linkage composition of the fibres has an important influence on fibre fermentation patterns. Fibres containing uronic acids induced the production of acetate, whereas fibres containing neutral constituent monosaccharides induced the production of propionate or butyrate. Fermentation of the fructans showed that molecular size could be an influential factor, and fermentation of complex heteroglycans showed that the arrangement of constituent monosaccharides in the molecules may also affect the fermentation patterns. This experiment also shows that monitoring of fibre degradation products is important for understanding how fibres are degraded during fermentation.

3.1. Introduction

Dietary fibres are resistant to digestion and absorption in the human small intestine, but they are fermented in the colon (1). The fermentation of dietary fibres results in gasses and short chain fatty acids (SCFA), such as acetate, propionate and butyrate (2). These SCFA are used by colonocytes as energy source or are absorbed and metabolised in the body (2, 3). Acetate and propionate may influence, for example, lipid and cholesterol metabolism (3) and butyrate is claimed to have various beneficial health effects, including the prevention of cancer and induction of satiety (4). The extent of SCFA production and the ratio between individual SCFA varies between types of dietary fibre (2).

The fermentation of dietary fibres in the colon is often simulated using *in vitro* fermentation methods. *In vitro* methods have the advantage over *in vivo* experiments that the methods are relatively simple (5). Moreover, a fibre can be tested without interference of other food components and low amounts of fibre are needed. These advantages outweigh the drawbacks of the method, such as the accumulation of fermentation products and the use of faecal inoculum, which might not be representative for the whole colon (6).

Animal models may overcome the latter drawback because they may allow the collection of digesta from the ileum or proximal colon for analysis as well as for inoculum. Compared to other mammals, pigs are the most suitable model for human *in vitro* fermentation studies because their colon is rather similar to human colon and most of the fermentation occurs in the colon (3). Pigs are monogastric omnivores, which allow their feed composition to be tailored to be similar to human food composition (7). Nevertheless, pigs may have a higher capability than humans to ferment dietary fibres because fermentation may already start in the terminal ileum and the colon microbiota includes many cellulolytic species (2, 8).

In vitro fermentation by pig and human inocula have been conducted in a number of studies, but direct comparison of the results is complicated by the differences in fibre sources, fibre concentration and experimental setups (5, 9) and (10). Fermentation of dietary fibres by faecal inocula from human and various animals including pigs has been studied only for the isolated fibres cellulose and citrus pectin and the complete cell wall mixtures citrus pulp and sugar beet pulp (11).

The aim of this experiment is to compare fibre fermentability by faecal microbiota from human and pig, and to reveal fibre properties which may influence fermentation kinetics and the formation of fermentation end-products. In this experiment, faecal microbiota from humans and pigs were used as inocula in *in vitro* fermentation studies on 12 isolated dietary fibres. During fermentation, besides cumulative gas production and SCFA production, the size and oligomeric profile of fibre degradation products were also monitored.

3.2. Materials and methods

3.2.1. Materials

Commercial grade dietary fibres were used in this experiment. Guar gum (Viscogum MP41230), alginate (Algogel 6020) and retrograded tapioca starch (C*Actistar 11700) were obtained from Cargill (Amsterdam, The Netherlands). Konjac glucomannan was from Kalys Agroalimentaire (Bernin, France). Cellulose (Vitacel[®] LC200) was from J. Rettenmaier & Söhne GmbH+Co (Rosenberg, Germany). Retrograded maize starch (Novelose 330) was from National Starch (Hamburg, Germany). Oat β -glucans (PromOat[™]) was from ScanOat[™] (Kimstad, Sweden). Inulin (Orafti[®] HP) and oligofructose (Orafti[®] P95) were from Beneo-Orafti (Oreye, Belgium). High methyl esterified (HM) citrus pectin (C74) was from CP Kelco (Lille Skensved, Denmark). Soy pectin (Soyafibe-S-DA100) was from Fuji Oil Co. Ltd. (Ibaraki, Japan), and xanthan gum was purchased from Sigma–Aldrich (St. Louis, MN, USA).

3.2.2. Faecal inoculum

Pig faecal inoculum was prepared from the faeces of three multiparous sows (Dutch Landrace). The pigs received no antibiotics and their body weights were between 264 and 368 kg. They were fed twice daily with a high fat (18.3 %(w/w)), low fibre (7.1 %(w/w)) diet (Table 3–1), that was composed to approximate the average composition of nutrient intake in adult humans according to the national food consumption survey in The Netherlands (12). The diet contained wheat (20 %(w/w)) and barley (20 %(w/w)) as fibre sources. Human inoculum was prepared from the faeces of three healthy donors. The diets of the donors were not controlled.

Table 3–1. Macronutrient composition of diet given to the pigs before faecal sampling.

Nutrient	Amount
Dry matter (g/kg)	904
Starch (g/kg)	416
Crude fat (g/kg)	183
Crude protein (g/kg)	147
Non-starch polysaccharides (NSPs) (g/kg)	71
Ash (g/kg)	69
Na (g/kg)	6.4
K (g/kg)	6.7
Cl (g/kg)	9.5
Ca (g/kg)	11.2
Digestible P (g/kg)	3.3
Gross energy (MJ/kg)	19.0
Digestible energy (MJ/kg)	16.9

The faeces were collected immediately after defecation in a plastic container previously flushed with CO₂ to maintain anaerobic condition. After collection, extra CO₂ was decanted over the container. Next, the container with faeces was closed and immediately put on ice. After pooling, the faeces were diluted six times (w/v) with sterilised 0.9 % (w/v) NaCl solution and subsequently homogenised with a blender for one minute. Next, the inoculum was filtered through two layers of cheesecloth and added into a fermentation medium with a ratio of 5:82 (v:v). Inoculum and fermentation medium were prepared according to Williams, et al. (13). Anaerobic conditions were maintained by flushing the liquids with CO₂. Liquids were kept at 39 °C. The inocula were prepared within 60 minutes after faeces collection.

3.2.3. *In vitro* fermentation

Fibres (337 mg) were weighed in duplicates into 250 ml screw cap bottles and flushed with CO₂. Fermentation medium with inoculum (60 ml) was added and the mixture was incubated in a waterbath at 39 °C. A blank sample without any fibre was used as a control of background fermentation. Gas production was measured continuously using a setup described by Cone, et al. (14).

A pilot study was conducted using pig inoculum to determine the sampling times for analysis in subsequent studies with inoculum from pigs and humans. In the pilot study, gas production was measured for 72 h. No samples were taken. Sampling times were defined as

the time when the gas production reached 1/3 of the maximum gas production (T_1), 2/3 of the maximum gas production (T_2), and when the maximum gas production was reached (T_3). The sampling times for each fibre are presented in Table 3–2. The experiments with pig inoculum and human inoculum were performed on separate days. At T_1 , T_2 , and T_3 of each fibre, samples were taken for further analyses.

Table 3–2. Sampling times for *in vitro* fermentation studies, as determined from a pilot study using a pig inoculum.

Fibre name	Fermentation time ¹ (h)		
	T_1	T_2	T_3
Guar gum	14.2	15.7	22.5
Konjac glucomannan	12.7	14.0	30.0
Cellulose	23.5	30.0	64.0
Retrograded tapioca starch	3.7	24.7	35.0
Retrograded maize starch	12.8	31.5	44.0
Oat β -glucan	3.4	5.1	15.3
Inulin	10.5	11.7	17.5
Oligofructose	5.5	7.0	17.5
HM citrus pectin	14.2	15.6	19.8
Alginate	22.9	25.6	42.0
Xanthan gum	9.4	11.7	31.0
Soy pectin	8.4	11.4	23.3

¹ T_1 : when 1/3 of the maximum cumulative gas production was reached. T_2 : when 2/3 of the maximum cumulative gas production was reached. T_3 : when the maximum cumulative gas production was reached.

3.2.4. Analytical methods

3.2.4.1. Dry matter and crude ash

For all fibres, dry matter was determined by drying to a constant weight at 103 °C, and crude ash was determined by incineration to a constant weight at 550 °C. Organic matter (OM) was calculated by subtracting crude ash from dry matter.

3.2.4.2. Short chain fatty acids (SCFA)

Acetate, propionate, and butyrate were analysed using gas chromatography, following the method described by Bosch, et al. (15).

3.2.4.3. Lactate

The samples were diluted with water (1:1 (v:v)) before analysis. The diluted samples (20 μ l) were injected into an Ultimate 3000 (Dionex Corporation, Sunnyvale, CA, USA) High

Performance Liquid Chromatography (HPLC) system coupled to a Shodex RI-101 refractive index detector (Showa Denko KK, Kawasaki, Japan), equipped with an Aminex HPX-87 H column (300 × 7.8 mm; Bio-Rad, Richmond, VA, USA) and a guard column. The eluent was 5 mM H₂SO₄, eluted at 40 °C with a flow rate of 0.6 ml/min.

3.2.4.4. Molecular mass distribution

Molecular mass (MM) distribution of the polysaccharides were analysed by High Performance Size Exclusion Chromatography (HPSEC) on an Ultimate 3000 HPLC system coupled to a Shodex RI-101 refractive index detector. The samples were diluted with water (1:1 (v:v)) before analysis and 20 µl were injected into the system. The separation was conducted through a series of TSK gel Super AW4000, AW3000 and AW2500 columns (150 × 6 mm) preceded by a guard column (Super AW-L). All columns were from Tosoh Bioscience (Tokyo, Japan). The eluent was 0.2 M NaNO₃, eluted at 40 °C with a flow rate of 0.6 ml/min. The molecular mass was estimated using a standard curve based on pullulan standards.

3.2.4.5. Oligosaccharide profiling

Oligosaccharide profiling was performed using an ICS 3000 High Performance Anion Exchange Chromatography (HPAEC) system (Dionex Corporation, Sunnyvale, CA, USA) with pulsed amperometric detection. The samples were diluted with water (1:1 (v:v)) before analysis and 20 µl was injected into the system. The separation was done using CarboPac PA-1 column (2 × 250 mm) preceded by a CarboPac PA-1 guard column (2 × 50 mm).

The same gradient was used for all samples in order to be able to compare chromatograms from different fibres. The monomers in the samples were eluted with a linear gradient of 0.02–0.05 M NaOH in 3 min and 0.05–0.075 M NaOH in 10 min, followed by isocratic elution of 0.1 M NaOH for 2 min. The oligomers were then eluted with a gradient of 0–1 M NaOAc in 0.1 M NaOH within 50 min. Subsequently, the column was washed with 1 M NaOAc in 0.1 M NaOH for 7 min, followed by 0.1 M NaOH for 3 min. Equilibration of the column was done by eluting 0.02 M NaOH for 20 min. The flow rate was 0.3 ml/min.

3.2.4.6. Constituent monosaccharide composition

Different methods were used for constituent monosaccharide composition analysis, depending on the type of fibre. Guar gum, konjac glucomannan, cellulose, retrograded

starch and β -glucan were prehydrolysed in 72 % (w/w) sulfuric acid at 30 °C for 1 h followed by hydrolysis in 1 M sulfuric acid at 100 °C for 3 h. The monosaccharides were subsequently derivatized into their alditol acetates and analysed using gas chromatography (16). Inositol was used as internal standard.

Fibres containing uronic acids (alginate, HM pectin, soy pectin, and xanthan gum) were analysed using the methanolysis method followed by trifluoroacetic acid (TFA) hydrolysis (17). The monosaccharides were analysed using HPAEC with post column alkali addition (18).

Inulin and oligofructose were hydrolysed using a commercial fructanase from *Aspergillus niger* (Fructozyme[®] L, Novozymes, Bagsvaerd, Denmark). The hydrolysis was performed at 1 mg/ml fibre solution, in 0.05 M sodium acetate buffer pH 4.7 at 50 °C for 18 h. For 1 mg fibre, 10 μ l of Fructozyme[®] L was used. The enzymes were inactivated by boiling for 10 min. After 200 times dilution, 20 μ l of the digest was injected into an HPAEC system with pulsed amperometric detection. Fructose, glucose, and sucrose were eluted using a gradient of 0–0.15 M NaOAc in 0.1 M NaOH in 15 min, followed by 9 min washing with 1 M NaOAc in 0.1 M NaOH and 15 min equilibration with 0.1 M NaOH. Total fructan concentration (C_f) was calculated using the equation according to Stöber, et al. (19):

$$C_f = k \cdot (F_f + G_f)$$

in which F_f is total fructose released from the fructans, and G_f is total glucose released from the fructans. k is a correction factor for water uptake during hydrolysis, which is based on the average degree of polymerisation (DP). For inulin, the average DP was 10, yielding a k of 0.910. For oligofructose the average DP was 4, yielding a k of 0.925.

3.2.5. Statistical analysis

Cumulative gas production (CV) of each fibre was fitted to either a monophasic or a biphasic model (20). The equations for both models are, respectively:

$$CV = \frac{A}{\left(1 - \frac{T_{1/2}}{t-L}\right)^2} \quad \text{and} \quad CV = \frac{A_1}{\left(1 - \frac{(T_{1/2})_1}{t}\right)^{B_1}} + \frac{A_2}{\left(1 - \frac{(T_{1/2})_2}{t}\right)^{B_2}}$$

in which A is the asymptotic gas production (ml/g OM), B is the switching characteristic of the curve, $T_{1/2}$ is the time when half of the asymptotic gas production (A) is reached (h), and t is time (h). L in the monophasic model is lag time (h). The subscripts following the factors in the biphasic model described the first ($A_1, B_1, (T_{1/2})_1$) and the second ($A_2, B_2, (T_{1/2})_2$) phases of the model. The nonlinear least squares regression procedure (PROC NLIN, SAS Inst. Inc., Cary, NC, USA) was used for curve fitting.

All parameters were analysed for effects of fibres and inocula by the GLM procedure in SAS using the following model:

$$Y = \mu + I_i + S_j + (I \times S) + \epsilon_{ijk}$$

in which Y is the dependent variable, μ is the mean, I_i is the effect of inoculum i , S_j is the effect of fibre j , $I \times S$ is the interaction between inoculum and fibre, and ϵ_{ijk} is the residual error term. Minimum Significant Differences (MSD) between fibres were calculated using SAS program (SAS Inst. Inc., Cary, NC, USA).

3.3. Results

3.3.1. Fibre composition

The 12 fibres used in this research were grouped as mannans, homoglucons, fructans, polyuronides and complex heteroglycans, based on their constituent monosaccharide composition, regardless of the linkages between the constituent monosaccharides (Table 3–3). Soy pectin and xanthan gum were grouped as complex heteroglycans because they contained both uronic acids and neutral constituent monosaccharides.

The constituent monosaccharide compositions of the commercial fibres (Table 3–3) generally corresponded with our expectations. However, the homoglucons were not pure. The commercial cellulose contained 15 %(w/w) xylose. Retrograded tapioca starch, retrograded maize starch, and oat β -glucan contained 47 %(w/w), 70 %(w/w) and 55 %(w/w) easily digestible starch, respectively, according to the product information obtained from the suppliers.

Table 3–3. Analysed constituent monosaccharide composition (mol%) and total constituent monosaccharide levels of fibres used for the fermentation studies.

Fibre	(mol%)									%(w/w)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Fru	UA	Total
<i>Mannans</i>										
Guar gum	0	0	2	1	60	32	2	na	2	92
Konjac glucomannan	1	0	0	0	61	0	37	na	2	97
<i>Homoglucans</i>										
Cellulose	0	0	0	15	1	0	81	na	1	99
Retrograded tapioca starch	1	0	0	0	0	0	98	na	1	99
Retrograded maize starch	1	0	0	0	0	0	98	na	1	96
Oat β -glucan	1	0	1	2	0	1	94	na	1	87
<i>Fructans</i>										
Inulin	na	na	na	na	na	na	4	96	na	77
Oligofructose	na	na	na	na	na	na	10	90	na	77
<i>Polyuronides</i>										
HM citrus pectin	3	1	5	0	0	9	1	na	81	68
Alginate	0	1	0	0	0	0	0	na	99 ¹	78
<i>Complex heteroglycans</i>										
Xanthan gum	0	0	0	0	31	0	52	na	16	60
Soy pectin	5	3	26	7	0	39	3	na	17	101

Rha: rhamnose, Fuc: fucose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, Fru: fructose, UA: uronic acids.

na: not analysed.

¹ Quantified using a glucuronic acid standard, guluronic acid/mannuronic acid = 1.1.

3.3.2. Cumulative gas production

Cumulative gas production was measured continuously during fermentation. Gas production patterns were fitted to either a monophasic or a biphasic model, depending on which model fitted best. For each fibre, the same model was fitted for describing gas production kinetics by both inocula. Fibres in one group did not always fit to the same model, as was shown for homoglucans and complex heteroglycans (Table 3–4). For fibres with monophasic gas production, except for cellulose, the time to reach half of the asymptotic value ($T_{1/2}$) or lag time (L) of pig inoculum tended to be higher than the $T_{1/2}$ or L of human inoculum.

Table 3-4. Gas production parameters during the *in vitro* fermentation of various dietary fibres using inocula from humans and pigs.

Fibre	Curve type ¹	Human inoculum				Pig inoculum				MSPE			
		CV	A	B	T _{1/2}	L	CV	A	B		T _{1/2}	L	
<i>Mannans</i>													
Guar gum	mo	295 ^{cd}	282 ^{ab}	5.2 ^{ab}	4.8 ^{ab}	0.5 ^a	0.053	295 ^{de}	318 ^b	10.3 ^c	16.6 ^e	0.0 ^a	0.055
Konjac glucomannan	mo	294 ^c	299 ^{bc}	6.4 ^b	7.8 ^c	0.0 ^a	0.035	332 ^{de}	344 ^{bc}	10.6 ^{cd}	18.4 ^e	0.0 ^a	0.047
<i>Homoglucans</i>													
Retrograded tapioca starch ^{2,3}	bi 1	303 ^{cde}	160	4.2	10.3	nd	0.012	182 ^c	162	6.5	4.8	nd	0.015
	bi 2		163	4.6	10.5	nd		na	na	na	na	nd	
Retrograded maize starch ^{2,3}	bi 1	278 ^e	181	3.4	22.9	nd	0.017	166 ^{bc}	36	8.4	3.7	nd	0.033
	bi 2		139	2.4	5.9	nd		na	na	na	na	nd	
Cellulose ^{§§}	mo	4 ^a	na	na	na	na	na	290 ^d	311 ^b	2.2 ^a	12.0 ^d	1.6 ^a	0.009
Oat β-glucan	mo	312 ^{cdef}	312 ^{abcd}	5.5 ^{ab}	4.0 ^a	0.0 ^a	0.052	393 ^{fg}	421 ^{de}	3.9 ^{ab}	4.8 ^{ab}	0.9 ^a	0.047
<i>Fructans</i>													
Inulin	mo	333 ^{ef}	358 ^{ef}	5.0 ^a	6.1 ^b	0.0 ^a	0.052	344 ^{ef}	374 ^{cd}	15.5 ^d	11.3 ^{cd}	0.0 ^a	0.083
Oligofructose	mo	330 ^{def}	344 ^{de}	5.1 ^{ab}	4.2 ^a	0.0 ^a	0.030	424 ^g	430 ^c	7.5 ^{bc}	7.7 ^{bc}	0.0 ^a	0.073
<i>Polyuronides</i>													
HM Pectin ³	mo	344 ^f	385 ^f	6.4 ^{ab}	6.1 ^b	0.0 ^a	0.093	121 ^b	na	na	na	na	0.121
Alginate	mo	238 ^b	254 ^a	7.5 ^b	17.8 ^d	0.0 ^a	0.038	148 ^{bc}	166 ^a	1.2 ^a	3.6 ^a	20.5 ^b	0.035
<i>Complex heteroglycans</i>													
Xanthan gum ²	bi 1	303 ^{cde}	239	12.9	15.4	nd	0.021	28 ^a	23	4.8	5.9	nd	0.023
	bi 2		65	1.6	5.7	nd		5	5	66.8	270.9	nd	
Soy pectin	mo	308 ^{cde}	328 ^{cde}	3.8 ^a	4.5 ^{ab}	0.0 ^a	0.035	303 ^{de}	339 ^{bc}	3.3 ^{ab}	12.3 ^d	0.0 ^a	0.059

The gas production from the blank sample was 0.6 mL in 64 h. Within a row, bold values indicate significant differences between human and pig inocula ($P < 0.05$). Within a column, data with different letter superscripts indicate significant differences between fibres ($P < 0.05$). Models are based on Groot *et al.* (20). ¹mo: monophasic; bi 1: biphasic, first phase; bi 2: biphasic, second phase; CV: Cumulative gas production (mL gas/g organic matter); A: Asymptotic gas production (mL/g organic matter); B: Switching characteristic of the curve; T_{1/2}: Time required to reach half of the asymptote value (A) of gas production (h); L: Lag time (h); MSPE: Mean square prediction error; ²nd: lag time was not included in biphasic model. ³na: poor curve fitting because stationary phase was not reached, or almost no gas was produced.



The mannans (guar gum and konjac glucomannan) were well fermented by both inocula (294–332 ml/g OM). Fermentation of these fibres by the same inoculum resulted in similar gas production parameters (Table 3–4). The fructans were also well fermented by both inocula, but oligofructose was fermented at a higher rate (lower $T_{1/2}$) than inulin.

The fermentability of fibres in the other groups differed between individual fibre sources, depending on the inoculum. Pig inoculum poorly fermented retrograded starches (166–182 ml/g OM), polyuronides (121 and 148 ml/g OM) and xanthan gum (28 ml/g OM). In contrast, human inoculum well fermented these fibres (238–344 ml/g OM), but hardly fermented cellulose (4 ml/g OM).

3.3.3. Short chain fatty acids (SCFA) and lactate

Total SCFA, which is the sum of acetate, propionate, and butyrate, is often used as an indicator of fibre fermentability besides gas production. In this experiment, the pig inoculum tended to produce less SCFA than human inoculum for most of the fibres (Table 3–5). The pig inoculum produced the least total SCFA from xanthan gum (3.01 mmol/g OM) and human inoculum produced the least total SCFA from cellulose (2.61 mmol/g OM). Both inocula produced the highest amount of total SCFA from soy pectin (9.06–10.59 mmol/g OM).

The SCFA composition obtained for the samples taken at the end of fermentation (T_3) varied for individual fibres (Table 3–5). Fermentation of polyuronides resulted in high proportions of acetate (78–81 % (mol/mol total SCFA)), fermentation of mannans, fructans, soy pectin and cellulose resulted in relatively high proportions of propionate (30–46 % (mol/mol)), whereas fermentation retrograded starch, oat β -glucan, and fructans yielded 10–19 % (mol/mol) butyrate. The SCFA composition was also influenced by the inoculum (Table 3–5).

Table 3-5. Total short chain fatty acids (SCFA), SCFA composition and soluble sugars after the *in vitro* fermentation of various dietary fibres using inocula from humans and pigs.

Dietary fibre	T ₃ ¹ (hh:mm)	Human inoculum			Pig inoculum		
		Total SCFA (mmol/g organic matter)	Composition (acetate: propionate: butyrate)	soluble sugars (% of initial total sugar)	Total SCFA (mmol/g organic matter)	Composition (acetate: propionate: butyrate)	soluble sugars (% of initial total sugar)
<i>Mannans</i>							
Guar gum	22:30	8.94 ^{bcd}	49 : 46 : 5	2	7.17 ^{fg}	60 : 31 : 9	2
Konjac glucomannan	30:00	8.43 ^{bed}	63 : 32 : 6	2	7.71 ^g	60 : 35 : 6	1
<i>Homoglucans</i>							
Retrograded tapioca starch	35:00	7.61 ^b	63 : 24 : 13	na	4.47 ^{bc}	65 : 22 : 14	na
Retrograded maize starch	44:00	7.70 ^b	56 : 27 : 17	na	5.08 ^{cd}	61 : 27 : 12	na
Cellulose ^b	64:00	2.61 ^a	63 : 21 : 15	na	8.11 ^{gh}	50 : 44 : 6	na
Oat β-glucan	15:18	8.22 ^{bc}	51 : 39 : 10	2	5.81 ^{de}	59 : 22 : 19	2
<i>Fructans</i>							
Inulin	17:30	9.24 ^{def}	60 : 30 : 11	0	4.52 ^{bc}	53 : 34 : 13	0
Oligofructose	17:30	8.76 ^{bode}	51 : 39 : 10	0	6.46 ^{ef}	53 : 35 : 12	0
<i>Polyuronides</i>							
HM citrus pectin	19:48	9.26 ^{def}	80 : 14 : 7	2	4.01 ^{ab}	78 : 17 : 5	51
Alginate	42:00	9.79 ^{def}	81 : 11 : 8	3	6.58 ^{ef}	80 : 16 : 4	4
<i>Complex heteroglucans</i>							
Soy pectin	23:18	10.59 ^f	62 : 31 : 7	1	9.06 ^b	64 : 32 : 4	2
Xanthan gum	31:00	10.02 ^{ef}	76 : 20 : 4	15	3.01 ^e	67 : 22 : 11	38

Within a row, bold data indicate significant differences between human and pig inocula (P < 0.05).

Within a column, data with different letter superscripts indicate significant differences between fibers (P < 0.05)

¹ T₃: Time when the last sample was taken. na: Not analyzed because of fiber insolubility.



During fermentation, the SCFA composition was not constant. Fermentation by pig inoculum is taken as an example. Figure 3–1 shows that for most of the fibres, SCFA production was dominated by acetate (69–84 %) during the initial stage of fermentation (T₁). As the fermentation continued, the proportion of acetate decreased (Figure 3–1a) while propionate proportions generally increased (Figure 3–1b). Butyrate proportions increased only for oat β -glucan, retrograded tapioca starch and inulin (Figure 3–1c). Although its proportion decreased during fermentation, acetate was still the dominant SCFA (53–80 %) at the end of fermentation (T₃).

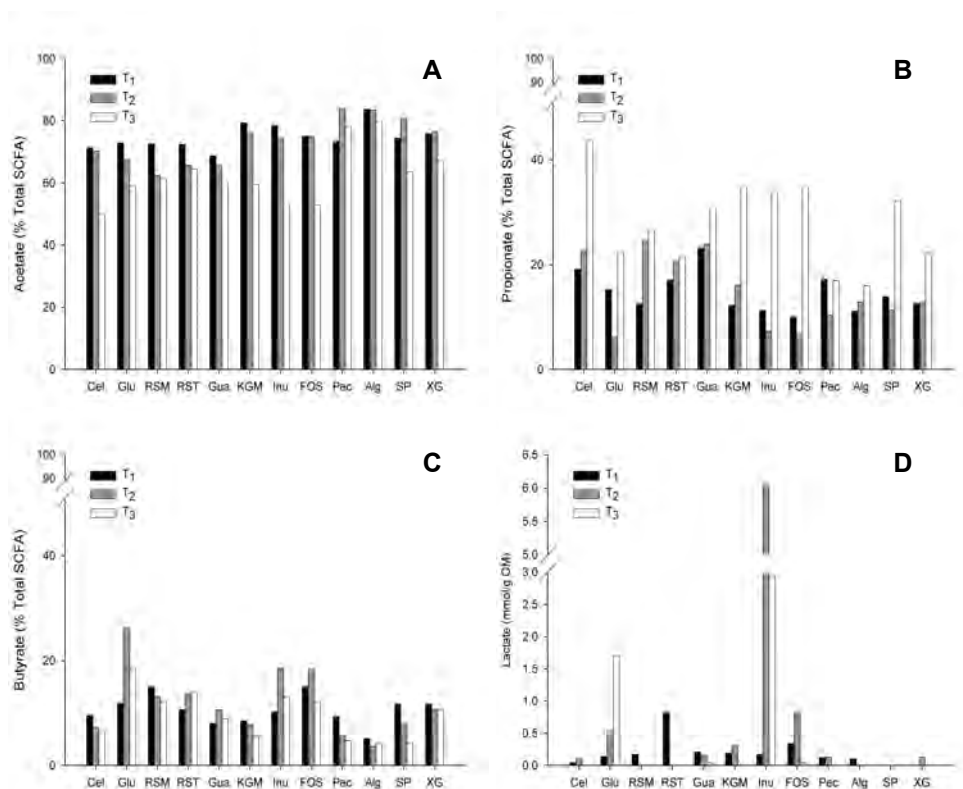


Figure 3–1. Levels of (a) acetate, (b) propionate, and (c) butyrate as a percentage of total short chain fatty acids (SCFA; acetate + propionate + butyrate) and (d) amount of lactate per gram organic matter in samples taken during the course of fermentation. T₁, T₂, and T₃ represent sampling times, which were determined as the time when the gas production reached 1/3, 2/3, and 3/3 of the maximum cumulative gas production, respectively. Samples were fermented by a faecal inoculum from pigs. Cel: cellulose, Glu: oat β -glucan, RSM: retrograded maize starch, RST: retrograded tapioca starch, Gua: guar gum, KGM: konjac glucomannan, Inu: inulin, FOS: oligofructose, Pec: citrus pectin, Alg: alginate, SP: soy pectin, XG: xanthan gum.

Lactate concentration was also monitored during fermentation (Figure 3–1d). Lactate was detected only in the early stage of fermentation, except for oat β -glucans and inulin where lactate was still present at the end of fermentation. For most of the fibres, lactate levels decreased between T_2 and T_3 , when propionate levels increased.

3.3.4. Fibre degradation during fermentation

For soluble fibres, the extent of fibre utilisation can be indicated by the amount of remaining soluble carbohydrates after fermentation. Except pectin and xanthan gum, the soluble fibres in this experiment were well fermented by both inocula with less than 5 % (w/w) of the fibres remained after fermentation (Table 3–5). For xanthan gum, the analysis of constituent monosaccharide composition of the remaining soluble carbohydrates showed that human inoculum did not ferment 13 % of all glucose, 16 % of all mannose and 14 % of all glucuronic acid. For pig inoculum, it was shown that 30 % of all glucose, 41 % of all mannose, and 54 % of all glucuronic acid were not fermented.

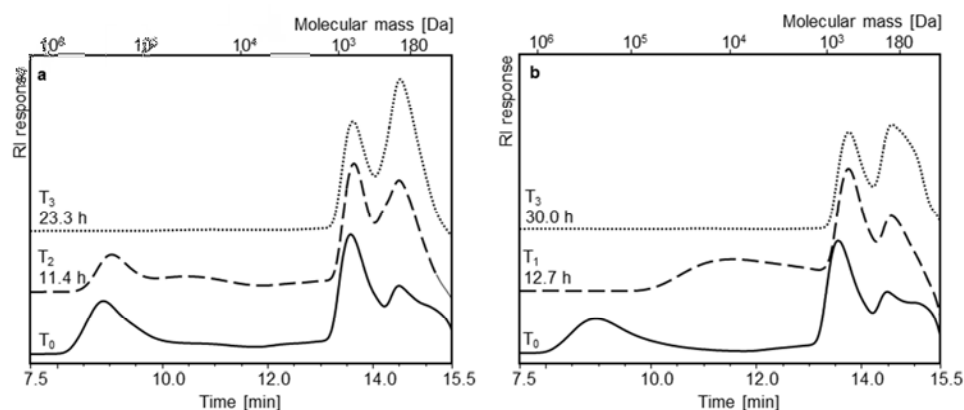


Figure 3–2. High performance size exclusion chromatography (HPSEC) elution patterns of (a) soy pectin and (b) konjac glucomannan after *in vitro* fermentation using faecal inoculum from pigs.

Fibre degradation products during fermentation were monitored for their MM distribution and oligomer profiles using HPSEC and HPAEC. The results for konjac glucomannan and soy pectin are presented as examples because they have different degradation patterns. The MM for both fibres at T_0 was approximately 2×10^5 Da (Figure 3–2). The compounds smaller than 10^3 Da are molecules, which were already present in the fermentation medium. After 11.4 h, only ~40 % of soy pectin molecules having a MM larger than 10^5 Da were

degraded. In a similar time span (12.7 h), all molecules of konjac glucomannan with MM higher than 10^5 Da were degraded.

The oligomeric degradation products were monitored by HPAEC (Figure 3–3). For soy pectin, at T_0 , there were a lot of monomers and oligomers (0–35 min). After 11.4 h (T_2), the amount of monomers and small oligomers declined, but a large variation of neutral and acidic oligomers was present (23–45 min). For konjac glucomannan, at T_0 there were not many monomers, but oligomers were present (24–28 min). After 12.7 h (T_1), the amount of oligomers increased. The poor resolution of the peaks showed that there were many different structures of similar sizes. At T_3 , nearly all oligomers from soy pectin and konjac glucomannan were utilised by the inoculum.

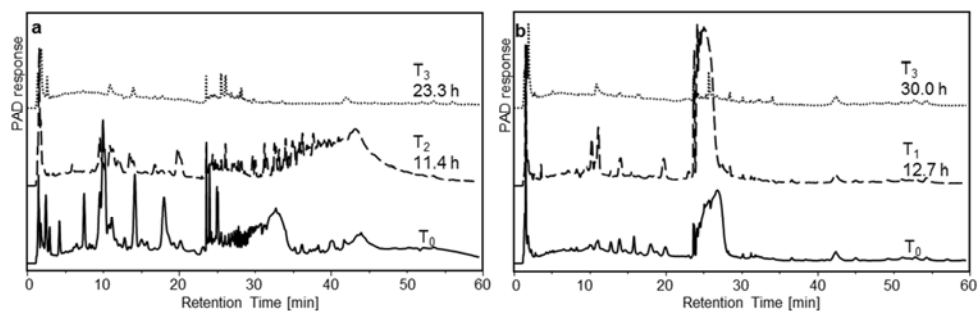


Figure 3–3. High Performance Anion Exchange Chromatography (HPAEC) elution profiles of (a) soy pectin and (b) konjac glucomannan after *in vitro* fermentation using faecal inoculum from pigs.

3.4. Discussion

3.4.1. Gas and SCFA production as indicators for fibre fermentability

In this experiment, the cumulative gas production and SCFA contents were monitored during fermentation. In general, both methods gave similar indications about the fermentability of the fibres. However, the fibres with the highest gas production were not always the highest in SCFA production and no strong correlation was found at the end of fermentation (T_3).

The low levels of SCFA, despite the high gas production for several fibres such as oat β -glucan when fermented by pig inoculum, could be partially explained by the presence of remaining lactate in the final sample (T_3). Lactate is an intermediate fermentation product,

which can be converted to propionate and butyrate (21). For oligofructose, however, there was very little (0.04 mmol/g OM) lactate in the sample. Another possibility for the lack of correlation between gas production and SCFA is that different fibres are fermented through different pathways, resulting in different proportions of gas and SCFA constituents. The latter hypothesis is supported by differences found in the SCFA composition upon fermentation of different fibres by both inocula.

Different levels of fermentability indicated by the gas production and SCFA contents suggested that to evaluate the absolute fermentability of dietary fibres, the two measurements of fermentation end products should be combined and supported by other parameters, such as intermediate products, remaining soluble carbohydrates or analysis of fibre degradation products.

3.4.2. Pig and human microbiota as inocula

Cumulative gas production, SCFA production and remaining soluble carbohydrate after fermentation shows that human inoculum was able to extensively ferment a broader variety of fibres than pig inoculum. This can be partly explained by the human gut microbiota being adapted to a large variety of fibres as a result of a heterogeneous and uncontrolled diet. The human diet contains diverse fibres including retrograded starch and pectins (1). On the other hand, the pigs were adapted to a diet with wheat and barley as fibre sources. These cereals contain little uronic acids (22). Because the microbiota composition seemed to be optimised for the fibres present in the diet, the pig inoculum had limited ability to ferment fibres containing uronic acids.

Interestingly, the human gut microbiota was not adapted to ferment cellulose although cellulose is present in various human food products. This agrees with the finding that even after two weeks of adaptation to cellulose, only 20 % of human respondents had faecal microbiota capable of degrading cellulose (23). It has also been reported that the pig microbiota contains more cellulolytic bacteria than human microbiota (8). Differences in the microbiota composition can also be caused by the genetic differences between the species, as well as between individuals (6).

3.4.3. Factors influencing the fermentation of dietary fibres

Fermentation of dietary fibres by one inoculum varied depending on constituent monosaccharide and linkage composition, as shown for mannans and homoglucons. The mannans generally showed similar fermentation patterns in gas production and SCFA production, whereas the homoglucons had clearly different patterns.

Both guar gum and konjac glucomannan could be degraded by the same (1,4)- β -D-mannanase (24). Therefore, it is likely that analogous types of microbiota, which produce (1,4)- β -D-mannanase were stimulated by the presence of these fibres. On the other hand, differently linked homoglucons (cellulose, retrograded starches and oat β -glucan) require different enzymes for their degradation. Therefore, it could be anticipated that different populations of microbiota are stimulated, which may affect the fermentation patterns.

Besides constituent monosaccharide and linkage composition, the DP also affects the fermentation, as exemplified by the fermentation of fructans. Although oligofructose (DP 2-8) is produced by partial hydrolysis of inulin (DP 2-60) (25), human inoculum produced SCFA with different compositions from these two fibres, whereas pig inoculum had different gas production parameters and total SCFA contents (Table 3-3 and Table 3-4). The different fermentation patterns between oligofructose and inulin are supported by Stewart, et al. (26). Shorter fibres can be degraded easily by exoenzymes, whereas longer fibres need both endo- and exoenzymes for optimal degradation. Because different enzymes were needed to utilise the two fibres, different groups of microbiota might be stimulated.

Contrary to the previously described fibres, which contain mostly neutral constituent monosaccharides, polyuronides are characterised by the presence of uronic acids (Table 3-3). Despite the differences in the chemical structures of the fibres and in the gas production and total SCFA production by the two inocula, the acetate proportions after fermentation of HM pectin and alginate were similarly high (78-81 % (mol/mol)). This was supported by separate studies with HM pectin (27) and alginate (28), although (11) found lower acetate proportions (57-59 % (mol/mol)) for citrus pectin.

The fermentation of complex heteroglycans (soy pectin and xanthan gum) was influenced not only by their constituent monosaccharide compositions, but also by the arrangement of the constituent monosaccharides in the molecule. Both fibres had comparable uronic acid contents (Table 3-3), but the fermentation patterns of soy pectin was similar to that of

neutral fibres, as shown by the gas production parameters and the relatively high (31–32 % (mol/mol)) propionate proportion. On the other hand, the fermentation of xanthan gum was more similar to that of polyuronides as shown by the high proportion (76 % (mol/mol)) of acetate produced.

In soy pectin, the uronic acid is located in the pectic rhamnogalacturonan backbone, which is protected by the long side chains of arabinan and galactan (29). In xanthan gum, the uronic acid is located in the trisaccharide side chain consisting of mannose and glucuronic acid, which is linked to every other glucose unit in a cellulosic backbone (30). These trisaccharide side chains protect the cellulosic backbone from enzymatic degradation (31). In both cases, the microbiota has to degrade the side chains before they have access to the backbone of the polysaccharides. For xanthan gum, early exposure to uronic acid may stimulate specific microbiota, which is characterised by high production of acetate, resulting in fermentation patterns similar to that of polyuronides. The utilisation of glucuronic acid by pig inoculum (46 %) was less than the utilisation of glucose (30 %) and mannose (41 %). This result suggests that pig inoculum poorly fermented xanthan gum because it has low ability to ferment uronic acids, as was also shown for HM pectin.

The fermentation of the 12 fibres revealed that constituent monosaccharide and linkage composition, DP and the arrangement of the constituent monosaccharides in the molecule affects how a fibre is degraded by a certain inocula and the composition of fermentation products.

3.4.4. Monitoring of fibre degradation products during fermentation

The structural changes of the fibre during degradation may influence fermentation patterns, including SCFA composition. In the early stages of fermentation, most of the fibres are present as polymers, which are not readily available as energy source. Therefore, the microbiota tended to produce acetate (Figure 3–1), because the conversion of constituent monosaccharides into acetate yields more energy than conversion into other SCFA (32).

The example of soy pectin and konjac glucomannan shows that monitoring of fibre degradation products is valuable for a more complete understanding of fibre degradation during fermentation. For these fibres, changes in MM distribution showed that up to 11–12 h of fermentation, soy pectin is more resistant to degradation than konjac glucomannan

(Figure 3–2). In contrast, the gas production results showed that soy pectin was fermented faster (lower $T_{1/2}$) than konjac glucomannan (Table 3–4).

Soy pectin is a highly branched polymer (29), which provides a lot of cleaving sites for exo-enzymes. Degradation by these enzymes results in monomers and dimers, which can be readily fermented by the microbiota. In contrast, konjac glucomannan is more linear, so that the microbiota needs to produce more endo-enzymes in the beginning of the fermentation. The degradation by endo-enzymes results in large oligomers, which cannot be used directly by the bacteria. Therefore, it is possible that the bacteria required more time to start fermenting konjac glucomannan than to start fermenting soy pectin, as indicated by the higher $T_{1/2}$ of gas production for konjac glucomannan than for soy pectin.

Our data allows the direct comparison of *in vitro* fermentation of 12 dietary fibres by faecal inocula from pigs and from humans. Human inoculum was able to ferment a broader variety of fibres than pig inoculum. The results also revealed that the fermentation of dietary fibres are influenced by the chemical characteristics of the fibre, which includes constituent monosaccharide and linkage composition, molecular size and the arrangements of the constituent monosaccharides in the molecule.

3.5. Acknowledgements

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In vitro fermentation of 12 dietary fibres by faecal inoculum from pigs and humans

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Chapter 4

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 constituent monosaccharide 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.

4.1. 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 (1). Resistant starch is a dietary fibre that has various health benefits, including improving bowel health, acting as a prebiotic, and increasing satiety (2).

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 (3) although the effects of resistant starch may depend on the types (4-6). 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 (7). 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 (8, 9), and to influence nutrient utilisation (10, 11).

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 (12). Resistant starch may modify the microbial composition in the large intestine. For example, resistant starch stimulated the growth of *Bifidobacterium spp.* in pigs (13) and increased the population of *Ruminococcus bromii* in humans (14, 15). 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 (16, 17).

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 constituent monosaccharide composition and enzyme activities in the large intestinal digesta.

4.2. Materials and methods

4.2.1. 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.

4.2.1.1. 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 m², 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.

4.2.1.2. Experimental diets

The compositions of the experimental diets are presented in Table 4–1. 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 were collected from the pigs.

Table 4–1. Composition of the experimental diets (g/100 g).

Ingredients	Digestible starch diet ¹	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 ²	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

¹The 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.

²The 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 (FeSO₄•H₂O); Cu: 10 mg (CuSO₄•5H₂O); Mn: 30 mg (MnO); Zn: 60 mg (ZnSO₄•H₂O); Co: 0.20 mg (CoSO₄•7H₂O); I: 0.75 mg (KI); Se: 0.20 mg (Na₂SeO₃).

4.2.1.3. 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.

4.2.2. 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.

4.2.3. Chemical and enzymatic analyses

4.2.3.1. 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, Schnellendorf, 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 (18), except that the overnight drying was performed at 50 °C instead of 80 °C. The dried sample was further analysed for carbohydrate content and constituent monosaccharide composition. Soluble NSP content and composition were calculated from the difference between total and insoluble NSP contents.

4.2.3.2. Constituent monosaccharide composition

Prehydrolysis was performed in 72 % (w/w) sulfuric acid at 30 °C for 1 h followed by hydrolysis in 1 M sulfuric acid at 100 °C for 3 h. After derivatisation into alditol acetates, monosaccharides were analysed using gas chromatography (19). Inositol was used as internal standard.

4.2.3.3. 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).

4.2.3.4. 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 \times 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 \times 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.

4.2.3.5. Protein content

Protein content of the extracts was measured by mixing 20 μ L of the sample or diluted sample with 200 μ L of Bradford reagent (20) 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 μ g/mL.

4.2.3.6. Analyses of enzyme activities

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 μ L of protein extracts and 200 μ L 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 μ M 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.

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; (21)) were treated with single component enzyme preparations containing endoarabinanase, arabinofuranosidase and galactanase (Novozymes, Bagsvaerd, Denmark) to remove the neutral side chains (pH 5.0 \pm 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 \times 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 carbohydrates in the enzyme digests and blanks was quantified using 4-hydroxybenzoic acid hydrazide (PAHBAH) method adapted for microtitre plate (22). Standard curves (0–250 µg/mL) of monosaccharides 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 carbohydrates (nmol) released in 1 min by enzymes extracted from one mg dry digesta.

4.2.4. 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.

4.3. Results and discussion

4.3.1. Constituent monosaccharide 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 4–2). The NSPs in the diet originated from wheat and barley. The main NSPs in both cereals are arabinoxylan, cellulose and mixed-linkage β -glucans (23). Only 25 % of NSPs in DS diet and 23 % of NSPs in RS diet were soluble. The glucose moieties in soluble NSPs were mainly from mixed-linkage β -glucans.

Table 4–2. Constituent monosaccharide composition of total non-starch polysaccharides (NSPs) in the experimental diets.

	g/100 g dry weight								Total
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	
<i>Digestible starch diet</i>									
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) ¹	0.3	7.5
<i>Resistant starch diet</i>									
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) ¹	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.

¹Numbers in brackets are mixed-linkage β -glucans.

For the large intestinal digesta, only results of arabinose, xylose, and glucose are presented (Table 4–3) as other constituent monosaccharides 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.

Table 4–3. Constituent monosaccharide composition of digesta from different parts of pig large intestine.

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

Data with different superscripts 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.

¹ 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.



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, et al. (24).

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 NSPs. 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 (25). 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 (26).

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 carbohydrates in the digesta was measured separately (Table 4–4). 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 (27). It has also been reported that some gut microbes, such as

Bacteroides thetaiotamicron, has enzymes capable of degrading starch molecules to smaller molecules which are then absorbed into the cell for further utilisation (28).

Table 4–4. Constituent monosaccharide 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	0.03 ^{a*}	0.00	0.03 [*]	0.01	0.33 ^{a*}	0.12	0.64 ^{a*}	0.19
	pCol	0.02 ^b	0.00	0.02	0.01	0.13 ^b	0.02	0.37 ^b	0.02
	pmCol	0.02 ^{ab*}	0.00	0.02	0.00	0.07 ^{b*}	0.01	0.32 ^{b*}	0.03
	dmCol	0.02 ^{ab}	0.00	0.02 [*]	0.00	0.06 ^{b*}	0.00	0.29 ^{b*}	0.01
	dCol	0.02 ^{ab}	0.01	0.02	0.01	0.05 ^{b*}	0.01	0.29 ^{b*}	0.06
RS	Cae	0.10 ^{a*}	0.06	0.17 ^{a*}	0.12	2.86 ^{a*}	0.80	3.69 ^{a*}	1.00
	pCol	0.02 ^b	0.01	0.03 ^b	0.02	0.76 ^b	0.58	1.34 ^b	0.63
	pmCol	0.01 ^{b*}	0.00	0.02 ^b	0.01	0.26 ^{b*}	0.08	0.76 ^{b*}	0.15
	dmCol	0.01 ^b	0.00	0.01 ^{b*}	0.00	0.20 ^{b*}	0.06	0.70 ^{b*}	0.17
	dCol	0.02 ^b	0.02	0.02 ^b	0.02	0.18 ^{b*}	0.04	0.61 ^{b*}	0.14

Data with different superscripts 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.

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 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 4–4, P-value <0.10), although there was no difference in total arabinose and xylose contents between treatments (Table 4–3). 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 (15), which did not present data on monosaccharide constituents.

In summary, results of constituent monosaccharide 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 (29). 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 were extracted and their activities were measured.

4.3.2. 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 4–5). Table 4–5 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 (30).

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 (31). 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 4–5. 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	9.2 ^{a*}	0.9	20.7 ^a	1.6	29.9 ^a	2.4
	pCol	7.2 ^{b*}	0.7	16.0 ^{a*}	3.2	23.2 ^{b*}	3.4
	pmCol	5.3 ^c	1.4	7.3 ^{b*}	3.3	12.6 ^{c*}	4.6
	dCol	2.4 ^{d*}	0.5	5.6 ^{b*}	3.2	8.0 ^{c*}	3.7
RS	Cae	10.6 ^{a*}	0.4	22.9	9.5	33.5 ^{ab}	9.2
	pCol	10.5 ^{a*}	1.7	28.1 [*]	6.3	38.6 ^{a*}	5.1
	pmCol	7.5 ^a	2.4	26.7 [*]	3.5	34.3 ^{ab*}	5.6
	dCol	4.0 ^{b*}	0.5	20.1 [*]	2.4	24.1 ^{b*}	2.9

Data with different superscripts 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.

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 (31), the amount of these enzymes was expected to increase as the microbial growth was stimulated by resistant starch in the diet.

4.3.3. Enzyme activities in the digesta

In an *in vitro* model, the enzymes produced by human faecal microbiota were influenced by the substrates present (31). 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 4–6.

Table 4-6. Enzyme activity in large intestinal digesta of pigs (nmol/mg dry digesta/min).

Diet Part	Polysaccharidase-degrading enzymes													
	Glycosidases													
	α -glc	β -glc	β -xyl	α -ara	wax	β -glc	cmc	hmp	lmp	lbg	ssp	sta	osx	cel
DS	Mean	1.4^a	2.6^{a*}	1.6^{a*}	5.2^{a*}	3.7^a	0.8	2.1	2.2	1.7	6.2	31.1^{a*}	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
pCol	Mean	0.8^{ab}	1.9^{ab}	1.5^{a*}	3.4^{ab*}	2.8^a	0.7	2.1	2.0	1.7	3.2	12.7^b	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
pmCol	Mean	0.5^b	0.9^b	1.0^{ab*}	1.3^{bc}	1.6^b	0.7	1.9	2.0	1.6	2.5	9.3^b	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	0.4
dCol	Mean	0.2^b	0.4^b	0.6^{b*}	0.8^c	1.4^b	2.1^c	0.9	2.2	1.1	1.4	7.8^{b*}	0.6	0.6
	SD	0.1	0.2	0.2	0.2	0.8	1.9	1.0	1.1	1.5	2.3	1.9	0.4	0.7
RS	Mean	1.3^{ab}	0.9	0.6^{ab}	2.4^a	2.7	8.0^{ab}	1.5	1.4	2.6	2.0	10.7^a	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
pCol	Mean	1.6^a	0.9	0.9^a	2.0^a	2.9	9.7^a	0.8	2.2	2.9	1.7	3.4^{ab}	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
pmCol	Mean	0.8^{ab}	0.8	0.7^{ab}	1.2^{ab}	2.0	6.7^{ab}	0.7	2.4	2.5	1.5	4.2^{ab}	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
dCol	Mean	0.4^b	0.4	0.4^b	0.7^b	1.9	3.2^b	0.8	2.6	2.4	1.0	1.6^b	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

Data with different superscript 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, β -glc: β -glucans, 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.

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 4.3.1). 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 4.3.1). 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 (32). It has also been reported that resistant starch did

not influence the digestibility of insoluble NSPs in humans (15), 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.

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 (33). β -Galactans were known to be present in wheat endosperm in the form of arabinogalactan (34). 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 (Figure 4–1). 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.

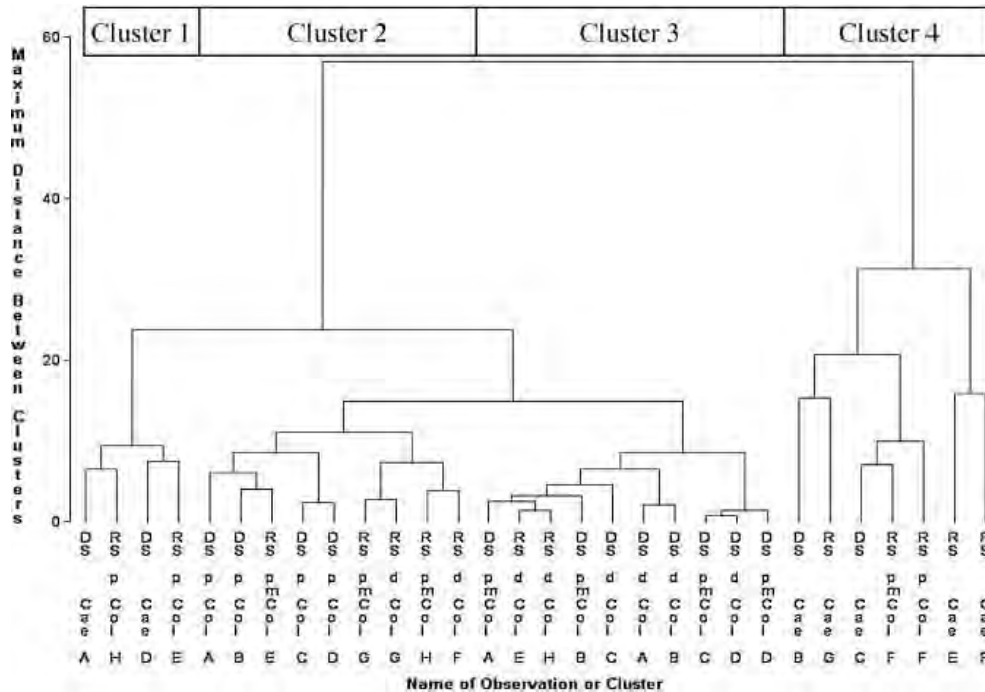


Figure 4-1. Cluster analysis of pig digesta based on enzyme activities towards 14 substrates. DS: digestible starch diet, RS: resistant starch diet, Cae: caecum, pCol: proximal colon, pmCol: proximal mid-colon, dCol: distal colon. A–D: pigs fed with DS diet, E–H: pigs fed with RS diet.

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 (3). 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.

4.4. Acknowledgement

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Chapter 5

Adaptation of growing pigs to diets rich in retrograded starch and/or alginate

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Abstract

This study aimed to investigate alginate degradation in the large intestine of pigs and the time required by the microbiota to adapt to alginate, in the presence and in the absence of retrograded starch. Experimental diets contained pregelatinised starch, retrograded starch, alginate, or a mix of retrograded starch and alginate. Faeces were collected at different time points. Up to day 39, the digestibility of alginate was limited (0.52 ± 0.10), and was lower with the inclusion of retrograded starch in the diet (0.34 ± 0.02). The digestibility of mannuronic acid (M) was 2-3 times higher than that of guluronic acid (G). Less than 10% of fecal alginate was water-soluble alginate oligosaccharides, whereas more than 90% of the fecal alginate had high molecular mass (~ 100 kDa) and was insoluble in water. The results show that the microbiota needed more than 39 days to adapt to alginate, thus adaptation time should be considered cautiously, especially when studying effects of dietary fibres at the distal parts of the colon.

5.1. Introduction

Alginate is a dietary fibre, which can be extracted from various seaweed, with multiple potential benefits to colon and cardiovascular health (1). Chemically, alginate consists of linear molecules of α -(1,4)-L-guluronic acid (G) and β -(1,4)-D-mannuronic acid (M) (2). A molecule of alginate consists of G-blocks, M-blocks, and MG-blocks with alternating M and G residues (3). The MG-blocks of several seaweed species were found to be shorter than the G-blocks (degree of polymerization (DP) >100), and the M-blocks (DP \geq 90) (4). The distribution of the different blocks in alginate determines its physical properties such as solubility (5), viscosity and gelling ability (6). Under acidic conditions (pH 2.85), G-rich alginate fraction are not soluble whereas M-rich alginate fraction is soluble (7). The G blocks in alginate may also interact with calcium and form a gel through an interaction that can be described by the 'egg-box' model (8). Detailed information on the chemical structure and physical properties of alginate is available in literature (9).

The physico-chemical properties of alginate determine its behavior in the digestive system. In the upper digestive system, alginate was reported to increase viscosity in the intestine (10) and reduce the absorption rate of certain nutrients in pigs (11) and humans (12). In humans, alginate with a high G:M ratio has a role in appetite regulation and enhancing satiety, which is related to the gelation of the G-rich alginate in the acidic environment of the stomach (13). In the colon, the presence of alginate may increase water content and the amount of faeces in humans (14). Depolymerised alginate with an average molecular weight of \sim 2000 was also shown to be potentially a prebiotic (15).

It has been reported that alginate was fermented slowly by human and pig fecal microbiota *in vitro*, resulting in an increased proportion of acetate compared to the fermentation products of other dietary fibres which contained neutral constituent monosaccharides (16). Among the many strains of microbiota present in the human colon that have been tested for their polysaccharide degrading activity, only *Bacteroides ovatus* has the ability to ferment alginate (17, 18). It was proposed that the ability of *Bacteroides* species to degrade various polysaccharides is inducible (19). Therefore, the rate of alginate fermentation of alginate might be higher when the microbiota is adapted to the substrate. When fermentation rate is increased, the caecum and proximal colon will become the more dominant sites of fermentation and less of the fibre will be available for fermentation in the distal part of the

colon. As a result, the fermentation at the distal colon can be lacking, depending on the amount and type of dietary fibres consumed. Lack of fermentation at the distal colon is unfavorable, because fermentation at the distal colon is considered beneficial for the prevention of various diseases (20).

By combining alginate with other dietary fibres which are easily utilised by the microbiota in the proximal large intestine, alginate fermentation is expected to be maintained in the distal colon. Resistant starch is regarded as a potentially suitable complement for alginate because it is rapidly fermented both in pigs (21) and humans (22). Also, it has been shown to be able to shift the fermentation of arabinoxylan-rich non-starch polysaccharides (NSPs) from wheat and barley to more distal parts of the large intestine (23). In addition, resistant starch has many other advantages for health, including stimulating the growth of beneficial bacteria in the colon and maintaining colon health (24).

The aim of the present experiment was to obtain more understanding on how alginate is degraded in the large intestine of pigs as models for human, and the time required by the microbiota to adapt to alginate, in the presence and in the absence of retrograded starch, as a source of resistant starch.

5.2. Materials and methods

5.2.1. Experimental setup

This study is a part of a larger experiment conducted at the experimental pig farm of Nutreco Swine Research Centre (Sint Anthonis, The Netherlands). Experimental protocols describing the management, animal care and sampling procedures were reviewed and approved by the Ethical Committee of Wageningen University (Wageningen, The Netherlands; DEC nr. 2011088.c).

The animals used for this experiment were growing gilts and barrows (Shade Duroc, Nutreco Swine Research Centre). Pigs were randomly assigned to dietary treatments balanced for body weight at birth and weaning, gender and litter. Initial body weight was approximately 25 kg and the body weight at the end of the experiment ranged between 82.8 and 140.4 kg.

5.2.2. Diets

The control diet (CON) contained carbohydrate from pregelatinised purified potato starch (Paselli™ WA5, AVEBE Food, Veendam, The Netherlands), wheat and soy meal. In the treatment diets, part of the pregelatinised starch was replaced with retrograded tapioca starch (C*Actistar 11700, Cargill, Amsterdam, The Netherlands) or with alginate (Pectacon M-5761, Acatris, Bunschoten, The Netherlands). The composition of the diets was calculated to be isoenergetic in gross energy (approximately 17 MJ GE/kg diet). The ingredient composition of the diets is presented in Table 5–1.

Table 5–1. Composition of the experimental diets (g/100g).

Ingredient	CON	RS	ALG	MIX
Pregelatinised potato starch	40.00	5.10	34.95	0.00
Retrograded tapioca starch	0.00	33.65	0.00	33.60
Sodium alginate	0.00	0.00	5.14	5.24
Soybean meal	35.00	35.73	34.95	35.67
Wheat	7.95	8.11	7.94	8.10
Wheat middlings	5.30	5.41	5.29	5.40
Animal fat	7.00	7.15	6.99	7.13
Vitamin and mineral premix ¹	1.00	1.02	1.00	1.02
CaCO ₃	1.25	1.28	1.25	1.27
Monocalcium phosphate	1.30	1.33	1.30	1.32
Salt	0.35	0.36	0.35	0.36
L-lysine HCL	0.15	0.15	0.15	0.15
DL-Methionine	0.20	0.20	0.20	0.20
L-threonine	0.10	0.10	0.10	0.10
TiO ₂ ²	0.25	0.26	0.25	0.25
Flavour (Luctarom Advance Cherry Honey)	0.15	0.15	0.15	0.15

Con: Control diet, RS: Retrograded starch diet, ALG: Alginate diet, MIX: Diet containing both resistant starch and alginate.

¹retinol, 6,000 IU; cholecalciferol, 1200 IU; DL- α -tocopherol, 40 mg; menadione, 1.5 mg; thiamin, 1 mg; riboflavin, 3 mg; D-pantothenic acid, 10 mg; niacin, 20 mg; cyanocobalamin, 15 μ g; folic acid, 0.2 mg; pyridoxine hydrochloride, 1 mg; choline chloride, 150 mg; Fe as Fe₂SO₄·H₂O, 80 mg; Cu as CuSO₄·5H₂O, 15 mg; Zn as ZnO₄·H₂O, 50 mg; Mn as MnO, 30 mg; Co as CoSO₄·7H₂O, 0.2 mg; I as KI, 0.7 mg; Se as Na₂SeO₃, 0.2 mg.

²TiO₂ was added into the diets for week 2-8.

5.2.3. Feeding

Before the experiment, all pigs were fed with a commercial basal diet. The pigs were then adapted to the pelleted experimental diets gradually within 5 days. Water and diets were available *ad libitum*.

5.2.4. Sampling

For every diet group, faeces were collected from two gilts. Rectal fecal “grab” samples were collected when the pigs were still fed with basal diet (day -7), followed by sampling on day 1, 3, 7, 14, 39 and 74 after they were fed solely with experimental diets. On day 39, the rectum of one of the CON-fed pigs was empty and no fecal sample could be collected. Samples were immediately stored at -20 °C until further analyses.

Diet samples were taken every batch. For analysis of Ti, the diet samples were analysed individually for the batches containing Ti, whereas for other analyses, diet samples from different batches throughout the experiment were pooled. The diets which contained Ti corresponded to the faeces from day 14 and 39.

5.2.5. Sample preparation

The pH and short chain fatty acids (SCFA) were measured in fresh faeces. For other analyses, the samples were freeze-dried and milled using ball-milling apparatus (MM2000, Retsch, Haan, Germany). Samples of the pelleted diets were milled passing 0.5 mm sieve using a milling apparatus (ZM200, Retsch).

5.2.6. Chemical analysis

5.2.6.1. Dry matter and pH

Dry matter was calculated from the weight of the sample before and after freeze-drying. The pH was measured after dilution of 500 ±5 mg of sample with 1 mL water.

5.2.6.2. Short chain fatty acids

Duplicate amounts of 500-600 mg sample were weighed and mixed with 5 mL 0.1 N sulfuric acid to inactivate enzymes and bacteria. An aliquot of the suspension was centrifuged (14,000 ×g, 5 min, 20 °C) and the supernatant was analyzed for SCFA using an Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA, USA). The SCFA were

separated using an isocratic elution of 0.01 N sulfuric acid at 0.6 mL/min for 30 min through an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Richmond, VA, USA) with a guard column. The columns were thermostated at 40 °C. The eluted compounds were monitored by a Shodex RI-101 detector (Showa Denko KK, Kawasaki, Japan). Acetic acid (0.2-2 mg/mL), propionic acid (0.2-2 mg/mL), butyric acid (0.2-2 mg/mL), isobutyric acid (0.05-0.5 mg/mL) and isovaleric acid (0.05-0.5 mg/mL) solutions were used as quantification standards. Results were expressed as the amount (μmol) of SCFA per gram of faeces.

5.2.6.3. Total starch

Total starch and its degradation products in the diets and in the faeces were analysed using a Total Starch Assay kit (Megazyme, Bray, Ireland). The starch was gelatinised in DMSO before being hydrolyzed with thermostable α-amylase and amyloglucosidase.

5.2.6.4. Constituent monosaccharide composition

For diet samples, the NSP constituent monosaccharide composition analysis was preceded by removal of starch according to a method described elsewhere (23), without defatting. All other samples were analysed for their constituent monosaccharide composition without any pretreatment.

Neutral constituent monosaccharides were measured by gas chromatography after pre-hydrolysis of the samples in 72 % (w/w) sulfuric acid at 30 °C for 1 h, hydrolysis in 1 M sulfuric acid at 100 °C for 3 h, and derivatization of the monosaccharides to their alditol acetates with inositol as an internal standard (25). For samples of diet ingredients, uronic acids in the hydrolysate were analysed using an automated colorimetric m-hydroxydiphenyl assay (26).

For analysis of alginate, the sample was treated in 80 % (w/w) sulfuric acid at 30 °C for 3 h. Hydrolysis was then performed in 2 N sulfuric acid at 100 °C for 2 h (27). The hydrolysate was diluted and injected into an ICS5000 High Performance Anion Exchange Chromatography system with Pulsed Amperometric Detection (HPAEC-PAD; Dionex Corporation, Sunnyvale, CA, USA) equipped with CarboPac PA-1 column (2 × 250 mm) and guard column (2 × 50 mm). The flow rate was 0.3 mL/min. The mobile phases were 0.1 M NaOH (A) and 1 M NaOAc in 0.1 M NaOH (B). The uronic acids were eluted using an isocratic flow of 20 %B for 10 min. After cleaning (20-100 %B in 5 min and 100 %B for

5 min), the column was re-equilibrated with 20 %B for 10 min before injection of the next sample. Glucuronic acid was used as quantification standard because guluronic acid and mannuronic acid were not available commercially.

Soluble carbohydrates were extracted by suspending 10 mg sample in 1 mL water followed by boiling for 15 min with frequent mixing. After centrifugation (14,000 ×g, 5 min, 20 °C), the soluble carbohydrates in the supernatant were subjected to methanolysis followed by hydrolysis in trifluoroacetic acid (28). The neutral monosaccharides in the hydrolysate were analysed using HPAEC-PAD with post-column addition (29), whereas the uronic acids were analysed using HPAEC-PAD using the gradient described above for alginate.

5.2.6.5. Identification of alginate oligosaccharides

The analysis of alginate oligosaccharides (AOS) in the soluble fraction of the faeces was conducted as described elsewhere (30). In short, the soluble AOS were extracted in boiling water and separated using a Thermo Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with an Acquity UPLC BEH Amide column (Waters Corporation, Milford, MA, USA). Detection of the AOS was performed using an MSⁿ detector.

5.2.6.6. Extraction of insoluble alginate

The water insoluble solid after extraction of the soluble fraction was freeze-dried. An aliquot of the freeze-dried water insoluble solid (60 mg) was mixed with 3 mL 50 mM NaOAc buffer pH 5.2 containing 50 mM EDTA and 50 mM ammonium oxalate. The extraction was conducted in a shaking water bath (70 °C, 2 h). After centrifugation (14,000 ×g, 5 min, 20 °C), the supernatant was collected and ethanol was added up to a final concentration of 80 %(v/v). The obtained suspension was centrifuged, and the pellet was subsequently washed with 80 %(v/v) ethanol and 96 %(v/v) ethanol, subsequently. After drying overnight in a vacuum oven at 50 °C, the extract was dissolved in water for analysis of molecular mass distribution.

5.2.6.7. Molecular mass distribution

Molecular mass distribution was analysed using an Ultimate 3000 (Dionex Corporation) HPLC system equipped with High Performance Size Exclusion Chromatography (HPSEC) columns (TSK gel Super AW4000, AW3000 and AW2500) and a Super AW-L guard column (Tosoh Bioscience, Tokyo, Japan). The temperature of the columns was kept at 55

°C. The elution was performed with an isocratic flow (0.6 mL/min) of 0.2 M NaNO₃ for 25 min. The eluted compounds were monitored by a Shodex RI-101 detector (Showa Denko KK) and by an Ultimate 3000 RS variable wavelength detector (Dionex Corporation) set at 280 nm to detect proteins. Pullulan standards (Sigma-Aldrich) were used for estimating the molecular mass of the compounds eluted.

5.2.6.8. Titanium

Titanium in the diet samples and in the faeces was analysed according to a modified method based on the method described by Short, et al. (31) and Myers, et al. (32). In principle, the samples were digested in concentrated sulfuric acid at 420 °C. After addition of hydrogen peroxide (30 % (v/v)), the absorbance at 408 nm was measured. Aliquots of 100 mg/L Ti were used as standards.

5.2.7. Apparent digestibility

The apparent digestibility (D) was calculated using the equation:

$$D = 1 - \left(\frac{Ti_0}{Ti_t} \cdot \frac{C_t}{C_0} \right)$$

Where Ti is the concentration of titanium and C is the concentration of the compound. The subscript 0 refers to the diet, and the subscript t refers to the faeces being analysed.

5.3. Results and discussion

5.3.1. Carbohydrate composition in the diet

The experimental diets contain carbohydrates from several sources. The main carbohydrate in the diets was starch and maltodextrins, which originated from the added pregelatinised and resistant starch sources (Table 5-1), as well as from wheat, wheat middlings, and soybean meal (Table 5-2). Wheat, wheat middlings, and soybean meal are also sources of non-starch polysaccharides (NSPs). Table 5-2 shows that the NSPs from these ingredients was dominated by neutral constituent monosaccharides. As a result, apart from alginate, more than 87 % (w/w) of the NSPs in the diet is composed of neutral constituent monosaccharides (Table 5-3). Only neutral NSPs will be discussed further in this paper.

Table 5–2. Constituent monosaccharide composition of the non-starch polysaccharide (NSP) sources in the diet (%(w/w)).

Ingredient	Starch	Constituent monosaccharide composition of NSP										Total NSP
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	G	M	
Soybean meal	4	0.3	0.3	2.2	1.2	1.5	7.7	8.3	2.7	n.a.	n.a.	24
Wheat	54	0.0	0.0	2.8	3.9	0.5	0.9	9.1	1.4	n.a.	n.a.	19
Wheat middlings	24	0.0	0.0	6.7	11.4	0.8	1.3	13.8	1.7	n.a.	n.a.	36
Alginate	n.a.	0.0	0.1	0.0	0.0	0.3	0.0	0.0	n.a.	71	31	103

Rha: rhamnose, Fuc: fucose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: non-alginate uronic acids, G: guluronic acid, M: mannuronic acid. n.a.: not analysed.

The alginate was composed of solely guluronic acid (G) and mannuronic acid (M). The G:M ratio was 2.3 with the assumption that the PAD responses of guluronic acid and mannuronic acid were similar. Analysis of alginate using glucuronic acid as a quantification standard resulted in an overestimation of alginate content (Table 5–2). The alginate in the diets, however, was underestimated (3.8 % and 4.2 % for ALG and MIX, respectively). Therefore, the amount and composition of alginate in the diet (Table 5–3) was calculated based on the ingredient composition of the diets (Table 5–1) and the constituent monosaccharide composition of alginate (Table 5–2).

Table 5–3. Carbohydrate composition of the experimental diets (g/100g).

Carbohydrate	CON	RS	ALG	MIX
Starch	42.1	41.5	38.0	36.6
Neutral NSPs	7.8	8.0	7.9	8.4
Non-alginate uronic acids	1.1	1.1	1.1 ¹	1.1 ¹
Alginate ²				
Guluronic acid	0.0	0.0	3.7	3.7
Mannuronic acid	0.0	0.0	1.6	1.6

CON: Control diet, RS: Retrograded starch diet, ALG: Alginate diet, MIX: Diet containing a combination of retrograded starch and alginate.

¹ Non-alginate uronic acids of alginate containing diets were assumed to be similar to the diets without alginate.

² The composition of alginate in the diet was estimated based on the ingredient composition of the diet and the constituent monosaccharide composition of the alginate.

Analysis results of the carbohydrate composition in the diet were used as a factor in calculating the apparent digestibility of the carbohydrates in the diet, which will be discussed in Sections 3.4 and 3.5.

5.3.2. Fecal dry matter

The fecal dry matter of all pigs fluctuated up to day 14 although they had been gradually adapted to the experimental diet for 5 days (Figure 5–1). It has been reported that mean total transit time of solid material in growing pigs (initial average body weight 30 kg) was 50 h for low fibre diet, and 41 h for high fibre (wheat bran) diet (33). Therefore, the faeces on day 1 might be influenced by the adaptation diet, which still partly contained the basal diet. The fluctuation between day 3 and day 14 indicate that within this period, the pig microbiota was adapting to the experimental diets. Castillo, et al. (34) reported that pig microbiota composition was unstable up to 21 days after the diet was changed.

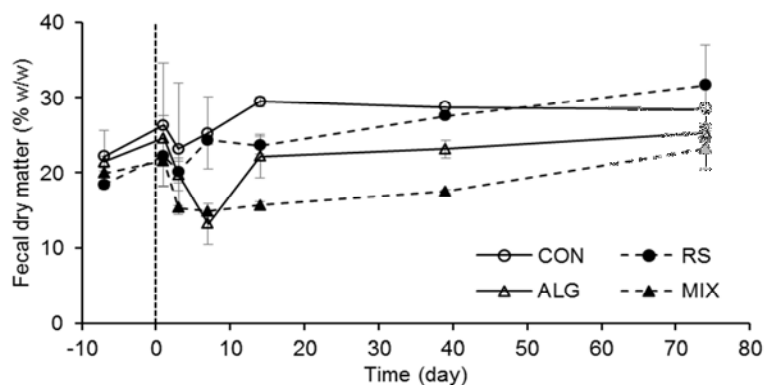


Figure 5–1. Fecal dry matter of pigs fed with control diet (CON) or experimental diets containing retrograded starch (RS), alginate (ALG), or a combination of retrograded starch and alginate (MIX). Data points are averages from 2 pigs, except for CON on day 39 (n=1). Error bars represent the standard deviation. At day -7, all pigs were fed a basal diet, from day -4 to 0 pigs were gradually adapted to experimental diets and from day 0 onwards pigs were fed solely their experimental diet.

From day 14 onwards, the CON-fed pigs had a relatively constant fecal DM content of approximately 30 % (w/w). Feeding the pigs with RS resulted in a fecal DM of 24 % (w/w) on day 14, which increased to similar (day 39) and slightly higher (day 74) values than CON. The ALG-fed pigs had a similar fecal DM (~22 % (w/w)) to RS-fed pigs on day 14, and the DM remained relatively constant up to 74 days. The MIX-fed pigs had the lowest fecal DM on day 14 (~16 % (w/w)), suggesting that the combined fibres had an

accumulative effect on fecal DM. After day 14, the fecal DM of MIX-fed pigs increased, and on day 74 it was similar to the fecal DM of ALG-fed pigs. The increasing fecal DM when resistant starch was present in the diets suggested that resistant starch influenced fecal DM only at early stage of adaptation, and that the effects of resistant starch on fecal DM diminished as the pigs became more adapted to the diets.

5.3.3. Fecal pH and SCFA

Changes in pH and SCFA concentration in the faeces are often used as indicators for fermentation of dietary fibres, especially when collection of the large intestinal digesta is not possible. The results of this experiment did not show prominent differences in fecal pH and SCFA between the treatments. The measured pH was 6.6 ± 0.4 and the total acetic acid, propionic acid and butyric acid content was 118 ± 23 $\mu\text{mol/g}$ faeces for all diets, with large variation between animals. Some other studies also reported no significant differences in fecal pH and SCFA concentration with the inclusion of dietary fibres in the diet (35, 36), although the consumption of dietary fibres is usually associated with increased SCFA in the large intestine (37).

It is possible that the effects on pH and SCFA were more pronounced in the proximal parts of the large intestine, and were not observed in the faeces, because up to 95 % of the SCFA produced by the microbiota can be taken up by the host (38). Moreover, It has been reported that the colon morphology of pigs changed after feeding with dietary fibres (39, 40). The change in colon morphology may lead to increased SCFA absorption, resulting in similar SCFA levels in the faeces despite the different diets consumed.

5.3.4. Degradation of starch and neutral NSPs

Besides fermentation indicators such as pH and SCFA, degradation of carbohydrates was monitored. The concentration of starch and its degradation products in the faeces were less than 5 % (w/w), and the apparent digestibility on day 14 and day 39 was above 0.995 for all samples (Table 5–4). This suggests that the starch in the diets, including retrograded starch, was utilised before reaching the rectum regardless of the presence of alginate in the diet. The high digestibility of starch on day 14 also indicated that the microbiota was able to extensively utilise resistant starch without a long adaptation time. Referring to the results

above, the lower fecal dry matter content for RS than for CON and for MIX than for ALG on day 14 (Section 5.3.2.) was not due to the presence of resistant starch in the faeces.

Table 5–4. Apparent digestibility¹ of carbohydrates in growing pigs fed with experimental diets (n=2).

Diets	Day	Starch		Neutral NSPs		Guluronic acid		Mannuronic acid		Total alginate	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CON	14	0.998	0.00	0.75	0.03	n.a.		n.a.		n.a.	
	39 ¹	0.998		0.71		n.a.		n.a.		n.a.	
RS	14	0.996	0.00	0.53	0.14	n.a.		n.a.		n.a.	
	39	0.998	0.00	0.68	0.01	n.a.		n.a.		n.a.	
ALG	14	0.996	0.00	0.68	0.04	0.28	0.12	0.72	0.01	0.41	0.08
	39	0.998	0.00	0.77	0.04	0.40	0.13	0.79	0.04	0.52	0.10
MIX	14	0.997	0.00	0.61	0.01	0.13	0.04	0.55	0.03	0.26	0.04
	39	0.999	0.00	0.72	0.01	0.22	0.02	0.61	0.00	0.34	0.02

CON: diet containing digestible starch, RS: diet containing retrograded starch, ALG: diet containing alginate, MIX: diet containing retrograded starch and alginate, n.a.: not applicable because the diets did not contain alginate.

¹Range of apparent digestibility: 0–1.

²n=1.

The digestibility of neutral NSPs is also presented in Table 5–4. For CON-fed pigs, the digestibility of neutral NSPs was approximately 0.75 on day 14. The digestibility of neutral NSPs in RS-fed pigs was lower than that in CON-fed pigs on day 14, with more variation between pigs for RS. The lower digestibility resulted in higher concentrations of neutral NSP in the faeces of RS-fed pigs (24 %(w/w), dry matter basis (dmb)) compared to the concentration in the faeces of CON-fed pigs (17 %(w/w), dmb) (Figure 5–2). These results suggest that the effect of RS on fecal dry matter was due to the lower digestibility of NSPs. It was previously reported that resistant starch may increase fecal weight by lowering the digestibility of NSPs and increasing the microbial biomass (22).

The fecal concentration of neutral NSPs seemed to be lowered by the presence of alginate in ALG and MIX (Figure 5–2), mainly because of the presence of alginate in the faeces, which diluted the other components. The neutral NSPs concentration in the faeces, therefore, was not indicative for the digestibility of neutral NSPs. The digestibility of neutral NSPs (Table 5–4) was lower for ALG-fed pigs than for CON-fed pigs on day 14,

but the effect was less than the effect found for RS. Alginate was not expected to be able to delay the utilisation of the neutral NSPs because it is known as a slowly fermented dietary fibre. Alginate has been reported to be able to inhibit proteases (1). It is possible, therefore, that some of the polysaccharide-degrading enzymes produced by the microbiota were inhibited by alginate, resulting in lower digestibility of neutral NSPs. With the inclusion of resistant starch in the diet, the digestibility of neutral NSPs for MIX-fed pigs on day 14 was lower than that for ALG-fed pigs, thus confirming that resistant starch was preferred by the microbiota over neutral NSPs and alginate.

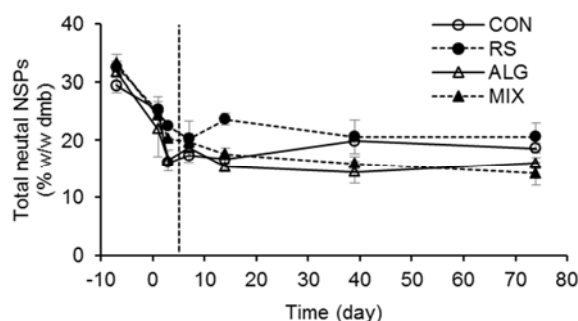


Figure 5-2. Concentration of neutral non-starch polysaccharides (NSPs) in the faeces of pigs fed with control diet (CON) or experimental diets containing retrograded starch (RS), alginate (ALG), or a combination of retrograded starch and alginate (MIX). Data points are averages from 2 pigs, except for CON on day 39 (n=1). Error bars represent the standard deviation. At day -7, all pigs were fed a basal diet, from day -4 to 0 pigs were gradually adapted to experimental diets and from day 0 onwards pigs were fed solely their experimental diet.

Between day 14 and 39, the digestibility of neutral NSPs for one of the CON-fed pigs was rather constant. There was no sample obtained from the other CON-fed pig on day 39. In contrast, the digestibility increased for RS-, ALG- and MIX-fed pigs. These results indicate that the CON-fed pigs were already adapted to the diet after 14 days, whereas the pigs fed with RS, ALG and MIX were still adapting. Pig microbiota was previously reported to take up to 6 weeks to be adapted to corn-based diet containing sugar beet pulp or wheat bran (34).

It was also observed that on day 39, the digestibility of neutral NSPs for RS-fed pigs was close to that of the CON-fed pigs. This substantiates the observation for fecal DM that the effects of RS on fecal parameters were limited to the early adaptation period, which probably occurred because after adaptation, resistant starch was fermented in the proximal part of the large intestine (23). In addition, the colon length of growing pigs might be

increased by resistant starch (41), so that the microbiota had enough opportunity to adapt to and utilise the remaining NSPs before it was excreted in the faeces.

5.3.5. Degradation of alginate

Alginate was present in significant amount (~20 % (w/w), dwb) in the faeces of both ALG-fed and MIX-fed pigs even after 74 days of adaptation (Figure 5–3A). The presence of alginate in the faeces may be related to the high fecal water content (1), as was described previously (Section 5.3.2). The relatively high amount of alginate in the faeces also indicated that the fermentation of alginate in the large intestine was limited. This was supported by the alginate digestibility, which shows that up to day 39, less than 55 % of the alginate in the diet was utilised by the microbiota.

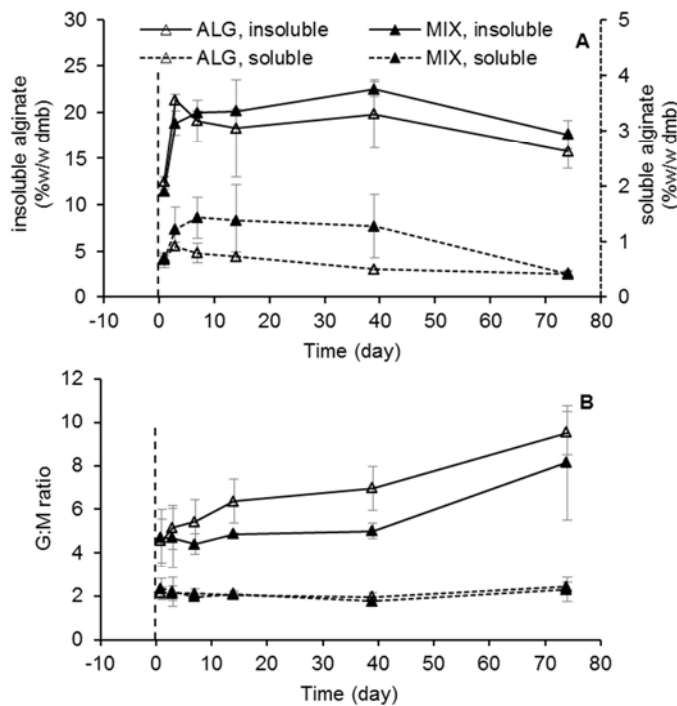


Figure 5–3. Concentration of insoluble and soluble alginate (A) and ratio of guluronic acid to mannuronic acid (G:M ratio) in the soluble and insoluble alginate (B) in the faeces of pigs fed with a diet containing alginate (ALG) or a combination of alginate and retrograded starch (MIX). Data points are averages from 2 pigs. Error bars represent the standard deviation. At day -7, all pigs were fed a basal diet, from day -4 to 0 pigs were gradually adapted to experimental diets and from day 0 onwards pigs were fed solely their experimental diet.

Alginate is composed of guluronic acid and mannuronic acid. In order to investigate the extent of utilisation of the two uronic acids, apparent digestibility was calculated separately for these uronic acids. Table 5–4 shows that M was more easily utilised by the microbiota than G. The preference of the microbiota to use M over G was also supported by the G:M ratio of alginate in the faeces (Figure 5–3B), which was higher than the ratio in the diet already on day 1. This shows that the microbiota was able to utilise M early during the adaptation period. The preferred utilisation of M to G by pig gut microbiota has been previously indicated (10), but the extent of utilisation was not quantified.

The digestibility of guluronic acid, mannuronic acid (Table 5–4), and the G:M ratio (Figure 5–3B), increased between day 14 and day 39, suggesting that the microbiota was still adapting after 14 days. With longer feeding time up to day 74, the G:M ratio increased further (Figure 5–3B). The changes between day 39 and 74 suggest that the microbiota was still adapting to alginate, even after 39 days. This finding further substantiates the long adaptation time needed by the gut microbiota after a change in the diet (34).

Comparison between the digestibility of alginate in ALG-fed pigs and in MIX-fed pigs indicated that the presence of resistant starch decreased the digestibility of both G and M in alginate at least up to day 39 (Table 5–4), and tended to increase the amount of soluble and insoluble alginate in the faeces (Figure 5–3A). Also, the G:M ratio for the insoluble alginate was lower when resistant starch was present (Figure 5–3B), indicating a less efficient utilisation of M in MIX-fed pigs compared to in ALG-fed pigs. These results point out that the inclusion of resistant starch in the diet altered the microbial composition, such that it had lower ability to utilise both soluble and insoluble alginate, as well as lower ability in utilizing M in the insoluble alginate.

5.3.6. Characterization of fecal alginate

In order to characterise alginate and its degradation products which were present in the faeces, the compounds in the faeces was fractionated based on their solubility in hot water.

5.3.6.1. Insoluble fecal alginate

More than 90% of the fecal alginate was insoluble in hot water. The G:M ratio of the insoluble alginate was relatively high. Alginate G-blocks can be precipitated in the presence of calcium or other divalent cations (8), which may inhibit its degradation by alginate lyase

(42). Therefore, it was attempted to solubilise the insoluble alginate with the aid of EDTA as a chelating agent. Nevertheless, only 40-60 % of alginate was extracted, suggesting that the insoluble alginate might also be associated with other compounds, such as proteins (43) or phenolic compounds (44).

The EDTA-extracted alginate had a molecular mass of ~100 kDa (DP >500; Figure 5–4). The alginate used in the diet had a molecular mass of approximately 225 kDa. It has been reported that the G-blocks from different species of algae can reach DP >100 (4). The EDTA-extracted alginate, therefore, was probably consisted of long G-blocks with short MG-blocks.

The molecular mass of the extracted alginate did not differ significantly between adaptation times (data not shown) and between diets (Figure 5–4). Thus, the M part of the insoluble alginate which was utilised over time as shown by the increase of G:M ratio were mainly located at the extremities of the molecule.

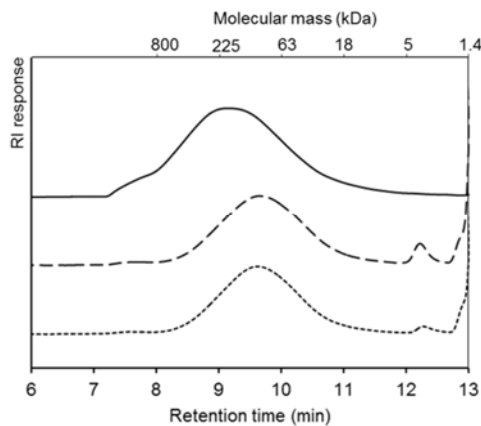


Figure 5–4. HPSEC elution pattern of extracted alginate from the faeces of pigs fed with a diet containing alginate (ALG) or a combination of alginate and retrograded starch (MIX) for 74 days. Data points are averages from 2 pigs.

5.3.6.2. Soluble fecal alginate

Less than 10% of the fecal alginate was soluble in water. Saturated and unsaturated AOS with different degree of polymerization (DP) could be identified using an UHPLC-MSⁿ method (30). Isomers with DP 2–4 could also be partially separated and putatively identified with the aid of the MSⁿ profile (45). Although quantification was not possible

due to the absence of standard AOS, the amount of AOS for the 2 treatments could be compared (Figure 5-5).

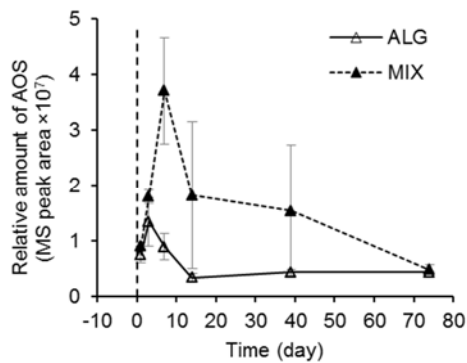


Figure 5-5. Relative amount of alginate oligosaccharides (AOS) DP 2-8 in the faeces of pigs fed with alginate (ALG) or a combination of retrograded starch and alginate (MIX). Data points are averages from 2 pigs. Error bars represent the standard deviation. At day -7, all pigs were fed a basal diet, from day -4 to 0 pigs were gradually adapted to experimental diets and from day 0 onwards pigs were fed solely their experimental diet.

The total AOS (DP 2-8) content in the faeces of MIX-fed pigs was higher than that of ALG-fed pigs, especially on day 7. The accumulation of AOS in the faeces of MIX-fed pigs on day 7 may suggest that the degradation of alginate took place in the distal colon and that the microbiota has not produced enough enzymes to utilise all of the AOS before they were excreted in the faeces. On day 74, the total AOS content of MIX-fed pigs was similar to that of the ALG-fed pigs, showing that between day 39 and 74 the microbiota was still adapting and utilizing AOS more efficiently in time. For MIX-fed pigs, individual variations were evident, shown by the large error bars. Detailed investigation on the individual variation is addressed elsewhere (30).

In summary, this experiment demonstrated that fermentation of a dietary fibre in the large intestine depends not only on the properties of the fibre itself, but also on the other dietary fibres present in the diet. Adaptation time to dietary fibres also has an important influence on the extent of fibre utilisation by the microbiota. As a result of adaptation, the effects of a fibre that were observed in the faeces after 14 days of adaptation might diminish when the adaptation time was extended to 74 days. Thus, adaptation time should be considered cautiously for future experiments involving dietary fibres, especially those aimed to observe effects at the distal parts of the colon.

For alginate, it was evident that although alginate is known as a soluble fibre, the G-blocks of alginate could be precipitated in the large intestine, thus hindering its utilisation by the microbiota even after a long adaptation time. Considering the higher digestibility of M compared to G, the results could have been different if a high-M alginate was used in the experiment. Heterogeneous degradation among dietary fibres within one category is also possible for other dietary fibres such as pectin, arabinoxylan, β -glucans, etc. Future experiments involving dietary fibres, therefore, should provide more detailed information about the chemical characteristics of the fibre.

5.4. Acknowledgements

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Chapter 6

Separation and identification of individual alginate oligosaccharides in the faeces of alginate-fed pigs

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Abstract

The research aimed to develop a method for analyzing specific alginate oligosaccharides (AOS) in a complex matrix such as pig faeces. The data obtained was used to study alginate degradation by the microbiota in the large intestine during adaptation, including the individual variation between pigs. A method using an UHPLC system with BEH amide column coupled with MSⁿ detection was able to distinguish saturated and unsaturated AOS with DP 2–10. Isomers of unsaturated trimer and tetramer could be separated and annotated. In the faeces, saturated and unsaturated AOS were present. The presence of unsaturated AOS indicates that the microbiota produced alginate lyase. The microbiota utilised unsaturated AOS more than saturated AOS. The results also suggested that guluronic acid at the reducing end of AOS inhibit the utilisation by microbiota during the first weeks of adaptation. After adaptation, the microbiota was able to utilise a broader range of AOS.

6.1. Introduction

Alginate is a linear polysaccharide that is composed of guluronic acid (G) and mannuronic acid (M) (1). As a dietary fibre, alginate is not absorbed in the small intestine and becomes a substrate for the microbiota, mainly in the large intestine (2). It has been shown that the digestibility of alginate by the microbiota in the large intestine of pigs (3) was limited compared to the digestibility of pectin (4), which is also mainly composed of uronic acids. Even after 39 days of adaptation to the alginate-containing diet, less than 60% of alginate was utilised by the microbiota in the large intestine (3). The remaining alginate was excreted in the faeces. Less than 10 % of the alginate in the faeces was soluble in hot water. This water-soluble fraction of alginate in the faeces may consist of alginate oligosaccharides (AOS).

In vitro, AOS may have various physiological effects towards different cells, such as human keratinocyte (5), human endothelial cells (6), and neuron-like cells (7). It has also been shown that AOS were able to interact with the immune system of mice *in vitro* (8) and *in vivo* through oral administration (9). Moreover, AOS were demonstrated to have prebiotic activity *in vitro* (10) as well as *in vivo* in rats (11). Additionally, AOS was able to inhibit colonization of the large intestine of chickens by the pathogen *Salmonella enteritidis* (12).

In most experiments, AOS used in the study were a mixture of unsaturated AOS produced from enzymatic degradation of alginate by alginate lyase. Such mixtures contain AOS with various degree of polymerization (DP) as well as isomers of AOS with the same DP. It was found that some effects were exerted by AOS with specific chemical structures (5, 6, 13). Therefore, it is of interest to develop analytical methodology for quantification of specific AOS in *in vivo* samples, such as intestinal digesta or faeces.

AOS resulting from alginate degradation in the large intestine are present in a complex matrix. Hence, to be able to investigate which specific AOS are present in digesta or faecal samples, a method that can analyse specific AOS in complex matrices is necessary. Ideally, the method should be able to separate AOS based on the degree of polymerization (DP) as well as separate isomers of the same DP.

Current methods for analyzing specific AOS often involve tedious purification of the specific isomers before analyzing those using MS and NMR (14, 15). Although this method provides the highest accuracy, it may not be applicable for biological samples because the amount of sample available for analysis is often limited and the concentration of AOS in the sample might not allow purification to single isomers.

Separation techniques, such as high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and capillary electrophoresis (CE) were able to separate AOS with different DP and isomers (16-20). Nevertheless, purified standards are necessary to be able to identify the compounds, and some compounds co-eluted (17). To overcome the need of purified standards and the interference by co-elution of different compounds, the separation methods can be coupled with mass spectrometry detection (MS^n). The MS^n detection allows identification of the compounds based on their molecular mass and fragmentation pattern. Hence, the use of MS enables the detection of compounds that co-elute, if they have different molecular masses. Unfortunately, the HPAEC methods used, which could separate AOS based on DP and isomers (17), are not compatible with the MS^n detector due to the high salt concentration used during analysis. Other LC- MS^n methods that has been applied for AOS includes the use of MS^n -compatible AEC (21), and reversed-phase liquid chromatography (RP-LC) (22), but the isomers were not separated (21) or were not identified (22).

Recently, LC- MS^n with porous graphitised carbon column (23) or with Ultrahigh Performance Liquid Chromatography (UHPLC) Ethylene Bridged Hybrid (BEH) amide column (24, 25) have been used to separate and identify various neutral and acidic oligosaccharides. For acidic oligosaccharides, such as pectins, the UHPLC-BEH amide column, which separates compounds based on the principle of hydrophilic interaction chromatography (HILIC), was able to separate unsaturated and saturated pectin oligosaccharides, as well as pectin oligosaccharides with different degree of methylation or acetylation (24, 25). The method used for pectins is assumed to be applicable for alginate, because both pectins and alginate have a backbone of uronic acids.

Hence, the present research aimed to develop an LC- MS^n method using an UHPLC-BEH amide column to separate and identify specific AOS in a complex matrix. It was also aimed

to analyse AOS present in the faeces of alginate-fed pigs, to understand how alginate is degraded by the microbiota in the large intestine of the pigs during adaptation.

6.2. Materials and methods

6.2.1. Preparation of alginate standards

A G-rich alginate and an M-rich alginate were obtained from SKW Biosystems (Rubi, Spain). The alginate (Pectacon M-5761) that was used for the *in vivo* study (3) was from Acatris (Bunschoten, The Netherlands).

The alginates were fractionated to obtain M-rich and G-rich alginate fractions using the method described by Haug, et al. (26). In short, the alginates were partially hydrolyzed in 1 M oxalic acid at 100 °C for 10 h. The fraction insoluble in the acid was solubilised at neutral pH, followed by pH adjustment to 2.85. At pH 2.85, the insoluble fraction (G-rich fraction) was subsequently separated from the soluble fraction (M-rich fraction) by centrifugation (1500 ×g, 10 min, 25 °C). After neutralization to pH 7.0, the alginate fractions were precipitated in 80 % (v/v) ethanol and dried overnight in a vacuum oven at 50 °C.

Unsaturated AOS were prepared by dissolving alginate or alginate fractions in water (5 mg/mL) and adjusting the pH to 7.0 ±0.2. Alginate lyase from *Sphingomonas* sp. (Megazyme, Bray, Ireland) was diluted to 500 µg protein/mL using 100 mM Tris buffer pH 7.2 containing 1 mg/mL BSA as was recommended by the supplier. An aliquot of 10 µL enzyme was added to 1 mL substrate solution. After incubation in a shaking incubator (40 °C, 24 h), the enzyme was inactivated by boiling for 10 min.

Saturated AOS were prepared by dissolving alginate or alginate fractions in water at 5 mg/mL. After adjusting the pH of the solution to 4.0 ±0.2 using HCl solution, the alginate was partially hydrolyzed at 120 °C for 3 h (27).

For analyses using UHPLC-MSⁿ, 100 µL of the 5 mg/mL solutions of AOS were diluted to 1 mg/mL by adding 25 µL ammonium formate buffer (eluent C in Section 2.3), 125 µL water, and 250 µL acetonitrile. After mixing, the mixture was centrifuged (14,000 ×g, 5 min, 20 °C), and the supernatant was used for analysis.

6.2.2. Extraction of AOS from pig faeces

Freeze-dried faeces were obtained from pigs fed with a diet containing retrograded starch and alginate. The experimental setup has been described elsewhere (3). The experimental protocols were reviewed and approved by the Ethical Committee of Wageningen University (Wageningen, The Netherlands; DEC nr. 2011088.c).

AOS were extracted by suspending 500 mg of freeze-dried faeces in 5 mL water, followed by boiling for 15 min with frequent mixing. After centrifugation (14,000 ×g, 5 min, 20 °C), the supernatant was collected, and the pellet was washed with 3 mL water. The supernatant from the washing was combined with the first supernatant, as crude extract of soluble materials.

The hydrophobic compounds in the extract were removed using Sep-Pak C18 cartridges (Waters Corporation, Milford, MA, USA), which had been activated with methanol and washed with water. The flow-through of the sample was collected, and cartridge was washed once with water. The washing was combined with the flow-through of the sample. After drying under a stream of air, the dried soluble compounds was re-solubilised in 4 mL of water.

An aliquot (225 µL) of this solution was mixed with 250 µL acetonitrile and 25 µL ammonium formate buffer (eluent C in Section 2.3). After centrifugation (14,000 ×g, 5 min, 20 °C), the supernatant was used for analysis using UHPLC-MSⁿ.

6.2.3. Analysis of AOS

The samples were analysed using an Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a Velos Pro ESI-Ion Trap-MSⁿ (Thermo Fisher Scientific). The separation was performed using an Acquity UPLC BEH Amide column (1.7 µm, 2.1 mm × 150 mm; Waters Corporation) preceded by a VanGuard BEH Amide precolumn (1.7 µm, 2.1 mm × 50 mm), which was kept at 35 °C. An aliquot of 5 µL sample was injected onto the column using an autosampler, while the sample tray was kept at 20 °C. Before injecting the sample, the needle was washed with 20 %(v/v) and 75 %(v/v) acetonitrile solution, as strong and weak wash, respectively (24).

The eluents used were water containing 1 %(v/v) acetonitrile (A), acetonitrile (B), and a buffer solution containing 200 mM ammonium formate, 2 %(v/v) formic acid, and 2 %(v/v)

acetonitrile (C). A gradient elution was used for analyzing the AOS. The buffer (eluent C) was constantly used at 5 % (v/v) of total eluent. The elution started with isocratic elution of 20 %A for 1 min, followed by a 50 min gradient from 20 %A to 45 %A to elute the AOS. The column was then cleaned from the remaining compounds using a gradient to 55 %A within 4 min and isocratic elution of 55 %A for 1 min. Re-equilibration of the column with 20 %A was performed for 14 min before injection of the next sample. The flow rate was constant at 500 $\mu\text{L}/\text{min}$, and a 1:9 ASI flow splitter (Analytical Scientific Instruments, El Sobrante, CA, USA) was used so that only 50 $\mu\text{L}/\text{min}$ of eluent went into the MSⁿ system for detection.

The MS detection was in negative mode, with source heater temperature at 225 °C and capillary temperature at 350 °C. The ion source voltage was -4.5 kV. The sheath gas, auxiliary gas and sweep gas flow were 33, 10 and 2, respectively (arbitrary units). The MSⁿ collection parameters was set according to the parameters described elsewhere (24), with the scan range of the MS set at m/z 300–2000. The data were processed using Xcalibur version 2.1.0 SP1 (Thermo Fisher Scientific).

6.2.4. Molecular mass distribution

Molecular mass distribution of alginate or alginate fractions were analysed using High Performance Size Exclusion Chromatography (HPSEC) as described elsewhere (3).

6.2.5. Alginate content, uronic acid composition, and apparent digestibility of alginate in pigs

The analyses of uronic acid composition of alginate, alginate content in pig faeces, and the calculation of the apparent digestibility were performed as described elsewhere (3).

6.3. Results and discussion

6.3.1. Uronic acid composition of reference alginate materials

The uronic acid compositions of alginate fractions obtained after partial hydrolysis in 1 M oxalic acid and precipitation at pH 2.85 of G-rich alginate, M-rich alginate and Pectacon alginate is presented in Table 6–1. The G-rich fraction with the highest G:M ratio (18.6) was obtained from G-rich alginate, and the M-rich fraction with the lowest G:M ratio (0.2)

was obtained from M-rich alginate. These fractions were used further as sources of AOS, to assist in the identification of AOS isomers produced from Pectacon alginate, which was the alginate used in the *in vivo* study (Section 6.3.3).

Table 6–1. Uronic acid composition of alginate and alginate fractions obtained from partial acid hydrolysis and separation by precipitation at pH 2.85.

Sample	mol%		G:M
	G	M	
M-rich alginate	49	51	1.0
Insoluble at pH 2.85	92	8	12.0
Soluble at pH 2.85 ¹	13	87	0.2
G-rich alginate	78	22	3.5
Insoluble at pH 2.85 ²	95	5	18.6
Soluble at pH 2.85	22	78	0.3
Pectacon M-5761	70	30	2.3
Insoluble at pH 2.85	95	5	17.9
Soluble at pH 2.85	18	82	0.2

G: guluronic acid, M: mannuronic acid.

¹ and ²: Fractions used for further analyses; ¹M-rich fraction; ²G-rich fraction.

6.3.2. Separation of specific alginate oligosaccharides

In order to obtain unsaturated AOS, the G- and M-rich alginate fractions and Pectacon alginate were degraded using alginate lyase. Saturated AOS were obtained by partial hydrolysis of alginates in HCl solution at pH 4.0. The extent of alginate degradation by the two depolymerization methods is illustrated by the change in molecular mass as analysed by HPSEC (Figure 6–1). The alginate and alginate fractions were degraded by acid, as shown by the lower molecular masses of the molecules in the acid hydrolysates compared to the molecular mass of the starting material. It was also shown that the extent of depolymerization by acid was similar for the three alginate and alginate fractions.

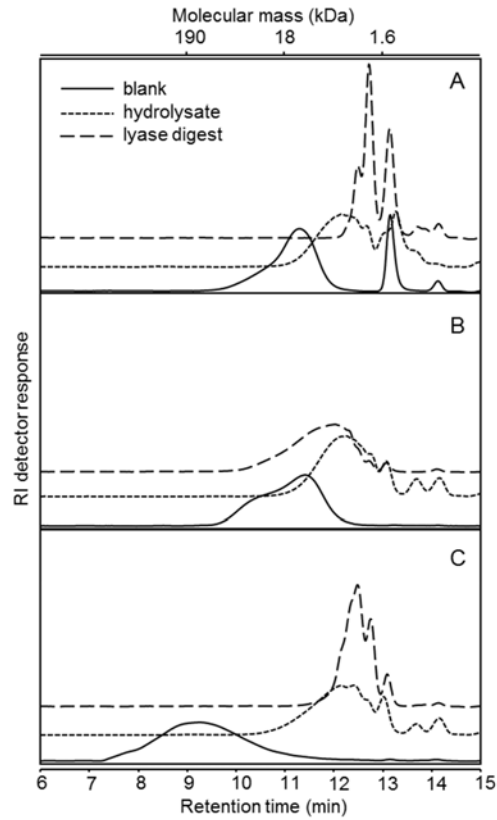


Figure 6–1. HPSEC elution patterns of M-rich alginate fraction (A), G-rich alginate fraction (B), and Pectacon alginate (C) before (blank) and after partial hydrolysis in acid at pH 4.0 (hydrolysate) or after digestion with alginate lyase (lyase digest). The upper x-axis shows the molecular mass based on calibration using pullulan standards.

Figure 6–1 also demonstrates that the alginate and alginate fractions were considerably degraded by alginate lyase. Pectacon alginate and M-rich alginate fraction was more degraded by alginate lyase than the G-rich alginate fraction (Figure 6–1B). This can be explained by the specificity of the commercial alginate lyase towards poly- β -D-mannuronate, as was described by the supplier. Nevertheless, the enzyme was not strictly specific, and part of the lyase digest of the G-rich alginate fraction had similar molecular mass with the hydrolysate. Therefore, AOS are expected to be present in lyase digests and hydrolysates of the alginate and alginate fractions.

The unsaturated AOS in the lyase digests and saturated AOS in the hydrolysates were then analysed by UHPLC-MSⁿ. The elution gradient that was previously developed for galacturonic acid oligomers (24) was modified to obtain a better separation between AOS isomers. Unsaturated and saturated AOS with DP 2-10 were identified based on the m/z values of the parent ions (Figure 6-2). Clusters of peaks with similar m/z values are isomers of AOS with the same DP. Figure 6-2 showed that unsaturated AOS eluted before saturated AOS with the same DP. This has also been found for unsaturated and saturated pectin oligomers analysed using BEH amide column, and was assumed to be due to the lack of the hydroxyl group at C4 position of the uronic acid residue at the non-reducing end of unsaturated pectin oligomers (24).

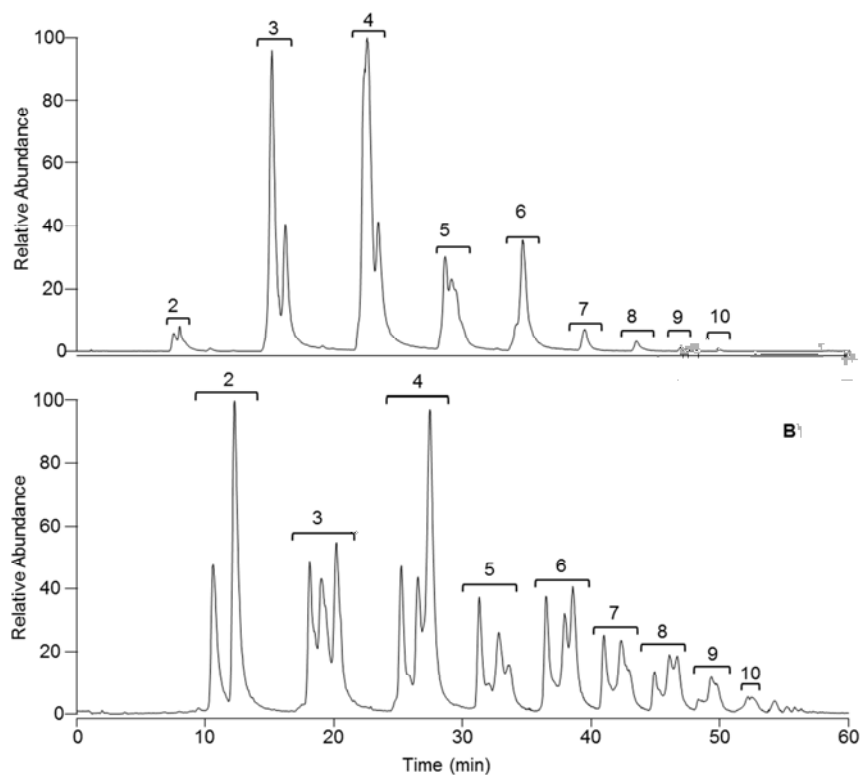


Figure 6-2. Elution patterns of unsaturated (A) and saturated (B) alginate oligosaccharide (AOS) separated by UPLC BEH Amide column coupled with MS detection. The AOS were obtained from Pectacon alginate after digestion by alginate lyase and after partial acid hydrolysis, respectively. The numbers denote the degree of polymerization of the AOS.

6.3.3. Building an alginate oligosaccharide UHPLC-MSⁿ library

The elution gradient used for AOS was able to partially separate isomers, especially those with DP 2-4, as indicated by the clusters of peaks with similar m/z values (Figure 6–2). Due to the unavailability of purified standards for each isomer, the isomers were identified based on the paper of Zhang, et al. (27), in which MS² spectra of different purified AOS were described. The MS² fragments were annotated according to the nomenclature described by Domon and Costello (28).

Identification of isomers was first attempted for the unsaturated trimers (Figure 6–3). Unsaturated AOS have a uniform non-reducing ends because 4-deoxy-L-erythro-hex-4-enopyranosyluronate (Δ ; ‘unsaturated uronic acid residue’) is formed at the non-reducing end regardless of the type of the uronic acid (29). Hence, there are 4 possible isomers of unsaturated trimer: Δ GG, Δ GM, Δ MG, and Δ MM. In the Pectacon alginate lyase digest, there seemed to be two isomers of unsaturated trimer, as shown by the two peaks in Figure 6–3A.

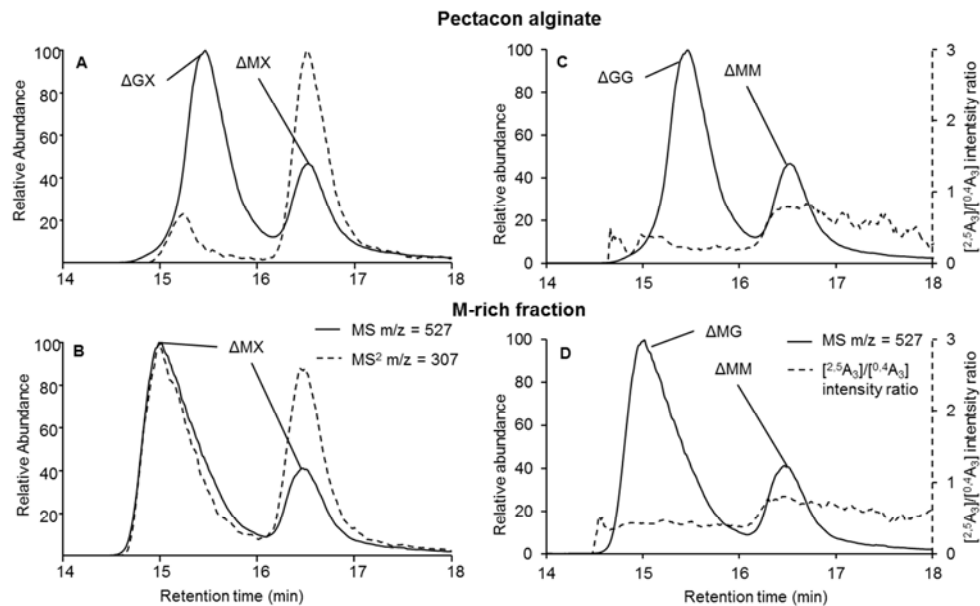


Figure 6–3. Zoomed-in UHPLC-MSⁿ patterns of unsaturated trimer isomers from Pectacon alginate and alginate fraction rich in mannuronic acid (M-rich fraction). The internal residues were identified based on the patterns of d-Z₂ (m/z=307; A,B). The reducing end was annotated based on the [^{2,5}A₃]/[^{0,4}A₃] intensity ratio (C,D). Δ : 4-deoxy-L-erythro-hex-4-enopyranosyluronate, G: guluronic acid residue, M: mannuronic acid residue, X: unknown uronic acid residue.

The identification of the internal residues was based on the presence of decarboxylated Z_n ions ($d-Z_n$) in the MS^2 spectra. It has been reported that an AOS with an M internal residue produced a fragment of decarboxylated Zn ions ($d-Z_n$), whereas an isomer with a G internal residue did not produce this fragment (27). For unsaturated trimer, the respective ion is $d-Z_2$ (m/z 307). Figure 6–3A showed that the trimer eluting at 16.53 min had the $d-Z_2$ fragment in its MS^2 spectra. Hence, it could be concluded that this trimer contained an internal M residue. In the MS^2 spectra of the other trimer, which was eluting at 15.47 min, the $d-Z_2$ fragment in its MS^2 spectra was also present, although the intensity was low. Nevertheless, the highest intensity was reached at a slightly lower retention time (15.24 min), indicating that there was co-elution of different isomers. By comparing the results with those of the lyase digest of M-rich alginate fraction (Figure 6–3B), it became clear that there is another trimer with an M internal residue eluting at around 15.02 min. As the retention times of the same compounds from the same sample can shift up to 0.3 min due to analytical variations, it is possible that the isomer in Pectacon digest that eluted at 15.24 min is the same as the isomer in the digest of M-rich alginate fraction that eluted at 15.02 min. Therefore, it could be concluded that the Pectacon alginate lyase digest contained 3 isomers of unsaturated trimer: two isomers with an M internal residue, one of which was present in low amount and partly co-eluted with the isomer containing a G-internal residue. The results illustrate that an overlay of the full MS base peak chromatogram for m/z 527 and the MS^2 base peak chromatogram for $d-Z_2$ (m/z 307) can assist the identification of co-eluting isomers when they have different internal residues.

After the internal residues were recognised, the reducing ends of the unsaturated trimers were annotated by comparing the intensities of internal fragmentation products $[^{2,5}A_3]$ and $[^{0,4}A_3]$, which have m/z values of 449 and 453, respectively. Zhang, et al. (27) reported that the ratio would be lower when the reducing end was G than when the reducing end was M. As the ratio is also influenced by the internal uronic acid residues, the method is best applicable for annotating isomers that differ only at the reducing end. By overlaying the ratio obtained at every time point with the Full MS base peak chromatogram for DP 3 (m/z 527), the reducing ends of the unsaturated trimer with M internal residue could be identified (Figure 6–3C, D). For the isomer with G internal residue (retention time 15.47 min), the $[^{2,5}A_3]/[^{0,4}A_3]$ ratio was low. Hence, the reducing end was annotated as G, representing an isomer of ΔGG (Figure 6–3C). Using this method, the 3 isomers of

unsaturated trimer could be identified. Δ MG eluted before Δ MM, suggesting that isomers with G at the reducing end elute earlier than similar isomers with M at the reducing end, as is summarised in Figure 6–4A.

A similar approach was used to annotate the isomers of unsaturated tetramer (Supplementary information, Figure 6–S1), but the annotation of the reducing end was more complicated because of the large overlap between the peaks (Figure 6–S1C,D). In this case, the information obtained from the trimers, that isomers with G at the reducing end may elute earlier, was used to annotate the reducing ends. As a result, four isomers of unsaturated tetramer were putatively identified (Figure 6–4B).

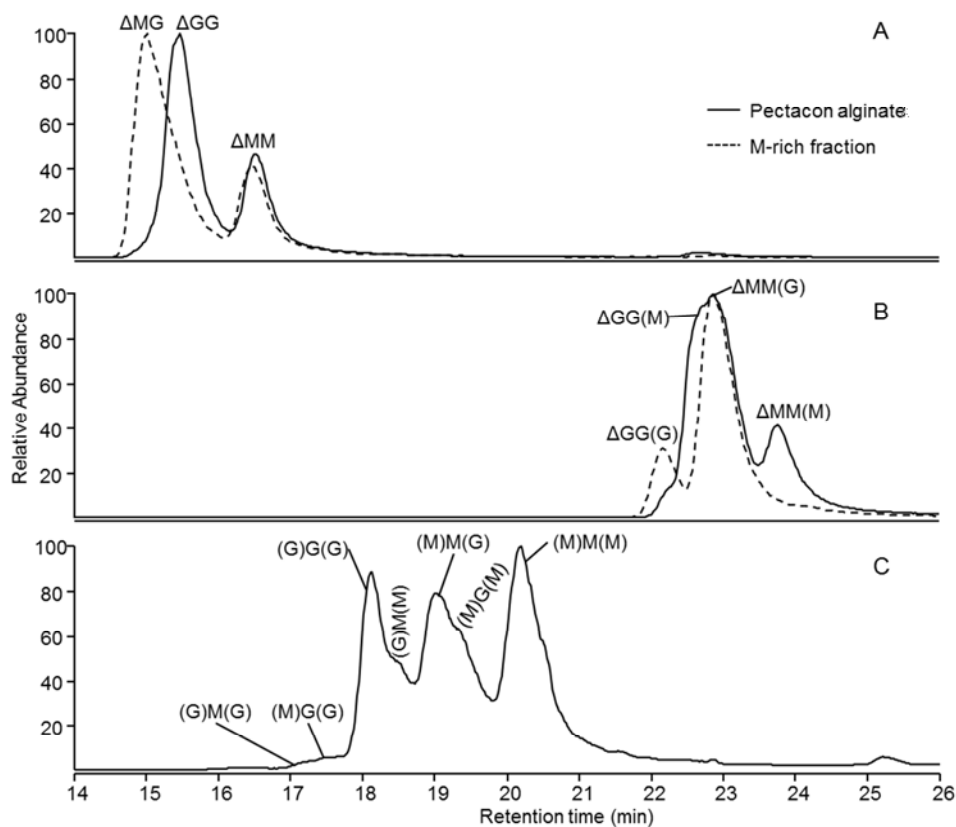


Figure 6–4. Summarised overview of the UHPLC-MSⁿ elution patterns of isomers of unsaturated alginate trimers, m/z 527 (A), unsaturated alginate tetramers, m/z 703 (B), and saturated alginate tetramers, m/z 545 (C) separated on UPLC BEH Amide column with online MSⁿ detection. Δ : 4-deoxy-L-erythro-hex-4-enopyranosyluronate, G: guluronic acid, M: mannuronic acid. The brackets indicate putative annotation of the uronic acid residue.

For isomers of saturated AOS trimer, the annotation was more challenging, because the non-reducing end can be either a G or an M residue. As a consequence, theoretically there can be 8 isomers for a trimer: GGG, GGM, GMG, MGG, MGM, MMG, GMM and MMM. Following the described approach, the internal residues were identified from the d-Z₂ fragment, and the reducing end as well as the non-reducing end was annotated putatively by comparing the [^{2,5}A₃]/[^{0,4}A₃] ratio, combined with the assumption that the presence of G decreases the retention time. In addition, the abundance of the isomers in G-rich alginate fraction or M-rich alginate fraction (Supplementary information, Figure 6–S2) was also taken into account. For example, an isomer of saturated AOS trimer that is abundant in G-rich fraction (G:M ratio 18.6) was expected to be rich in G. As a result, seven isomers in the acid hydrolysate of Pectacon alginate were putatively annotated. The elution behavior as well as the annotation of isomers of saturated trimer is summarised in Figure 6–4C.

Figure 6–4 shows that part of the peak clusters illustrated in Figure 6–2 was formed by isomers of AOS. Despite the inability to absolutely annotate each isomer peak, the analysis method described above was proven to be useful for analyzing specific AOS in complex mixtures. The information obtained and the AOS UHPLC-MSⁿ library was then applied for analyzing AOS in pig faeces, in order to understand the mechanism of alginate degradation in the large intestine of pigs.

6.3.4. Alginate oligosaccharides in pig faeces

In previous research, it was shown that the faeces of alginate-fed pigs contained 15-20 % alginate based on dry matter, even after 74 days of adaptation; with less than 10 %(w/w) of the alginate being water-soluble (3). An example of the elution patterns obtained from the water-soluble faecal extract is presented in Figure 6–5. In the base peak chromatogram (Figure 6–5A), a number of AOS could already be recognised. By filtering the base peak chromatogram for m/z values specific for AOS parent masses, it was shown that both unsaturated AOS (Figure 6–5B) and saturated AOS (Figure 6–5C) were present in the faeces.

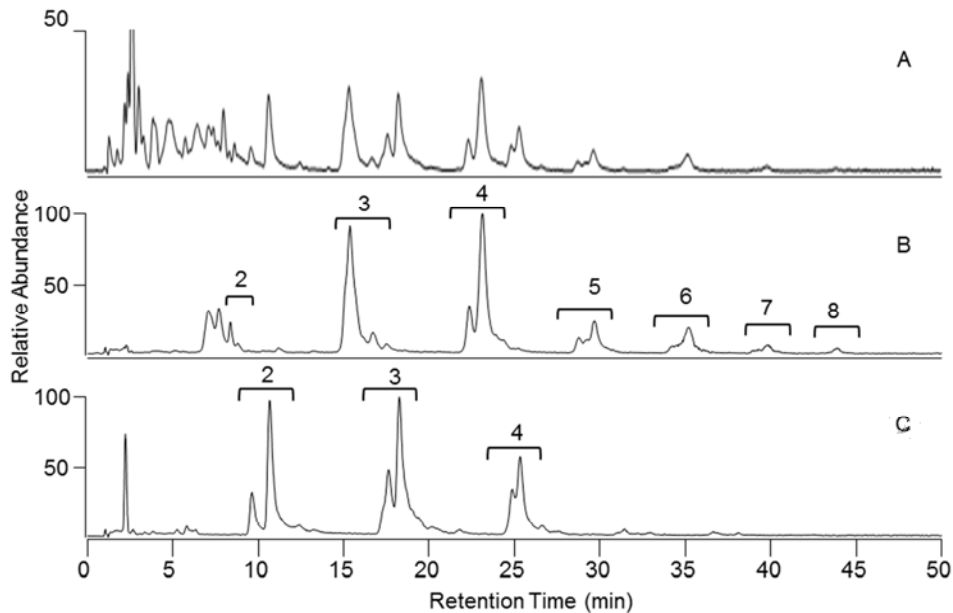


Figure 6-5. Elution patterns of alginate oligosaccharides (AOS) extracted from the faeces of an alginate-fed pig (Pig E, day 7), after separation by UPLC BEH Amide column coupled with MS detection. The base peak chromatogram (m/z 300–2000) (A) was filtered for the m/z values of unsaturated AOS (B) and saturated AOS (C) as necessary. The numbers denote the degree of polymerization of the AOS.

Assuming that the MS signal intensities are similar for DP 2-8, the total amount of AOS is reflected by the peak area of the AOS. For the comparison between samples from the same pig, the AOS peak area for a faecal sample from a given pig was compared relatively to the highest AOS peak area achieved for the same pig (Figure 6-6). For both pigs, the highest total AOS content in the faeces was achieved on day 7. The increase of total AOS content in the faeces on the first days of adaptation suggested that the microbiota was adapting to alginate, and alginate-degrading enzymes were produced. Nevertheless, after 74 days the total AOS content in the faeces of both pigs were similarly low, suggesting besides being able to degrade alginate to AOS, the microbiota was also able to utilise the AOS efficiently. Between day 7 and 74, there was individual variation between the pigs in alginate degradation, which will be addressed in more detail in Section 6.3.5.

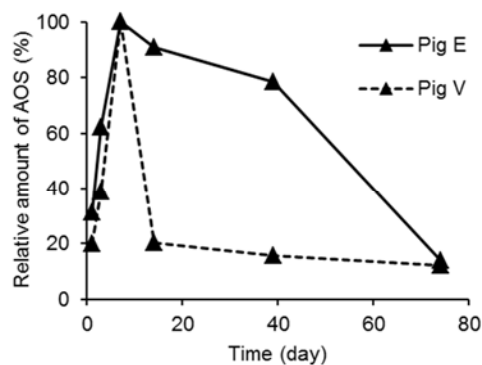


Figure 6-6. Relative amounts of AOS in the faeces of pigs during 74 days feeding with alginate-containing diet. The total AOS content in the faeces of a given pig is expressed in comparison to the highest total AOS content reached for the same pig, based on total AOS peak area as analysed by UHPLC-MSⁿ.

The same data obtained from the UHPLC-MSⁿ that was used to determine the total AOS content, was also used to quantify unsaturated and saturated AOS of different DP. Early in the adaptation period (day 1-3), the relative amount of unsaturated AOS was lower (pig E), or similar (pig V) to the amount of saturated AOS (Figure 6-7). As the pigs adapted to the diet, the proportion of the unsaturated AOS increased, with individual differences between pigs. The highest proportion of unsaturated AOS was reached on day 39 for Pig E, whereas it was reached on day 7 for pig V. After reaching its maximum, the proportion of unsaturated AOS declined gradually along the feeding period.

The increasing amounts of unsaturated AOS during the early period of adaptation (Figure 6-7) showed that the inclusion of alginate in the diet stimulated the production of alginate lyase by the microbiota in the large intestine of pigs. It has been reported that alginate lyase, which comprises the majority of alginate degrading enzymes (29), is also produced by rumen microbiota (30) and human large intestinal microbiota (31).

With the assumption that alginate lyases were the main alginate-degrading enzymes produced by the microbiota, the saturated AOS were formed only from the non-reducing end of every alginate molecule. Hence, saturated AOS were expected to be present in small amounts compared to unsaturated AOS. In contrast, on day 1-3 the amounts of saturated AOS were relatively high compared to unsaturated AOS (Figure 6-7). This indicates that before the microbiota was adapted to alginate, saturated AOS accumulated. With longer

adaptation period, the microbiota has more ability in utilizing saturated AOS, but the utilisation of saturated AOS was still lower than the utilisation of unsaturated AOS, as was shown by the increasing proportion of saturated AOS relative to that of unsaturated AOS between day 39 and day 74 for both pigs.

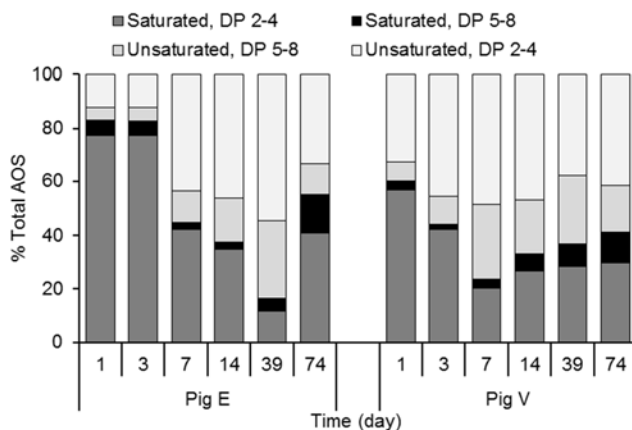


Figure 6-7. Proportions of unsaturated and saturated alginate oligosaccharides (AOS) with different degree of polymerization (DP) in the faeces of pigs during 74 days feeding with alginate-containing diet. The proportions are based on the total AOS peak area based on UHPLC-MSⁿ elution patterns.

By zooming in on the AOS elution patterns for the faecal samples (Figure 6-5) and comparing the retention times and the MSⁿ spectra of the AOS isomers that were present in the faeces with the retention times of putatively identified AOS (Section 6.3.3), the different isomers of saturated trimer, unsaturated trimer, and unsaturated tetramer of the AOS in the faecal samples could be annotated. Figure 6-8 depicted the identified AOS, which were present in the faecal samples on day 7. For both pigs, the dominant unsaturated trimer was Δ GG, followed by Δ MG (Figure 6-8A). The dominant unsaturated tetramers were Δ GG(G) and Δ MM(G) (Figure 6-8B). AOS composed of only M residues (Δ MM and Δ MMM) were present in very low amounts. From the results of the apparent digestibility (Table 6-2), it is shown that M was utilised more than G. Hence, it is concluded that the low amount of Δ MM and Δ MMM was a result of extensive utilisation and not because of a low digestibility of M by the microbiota.

The preferential utilisation of Δ MM and Δ MM(M) over Δ MG and Δ MM(G) up to day 7 indicates that the presence of G at the reducing end inhibits the utilisation of the AOS in a non-adapted situation. This was also supported by the remaining saturated trimers (Figure



6–8C). Nevertheless, after a longer adaptation period up to 74 days, there was no accumulation of specific AOS (results not shown), which means that after adaptation the microbiota was able to utilise a broad variation of AOS.

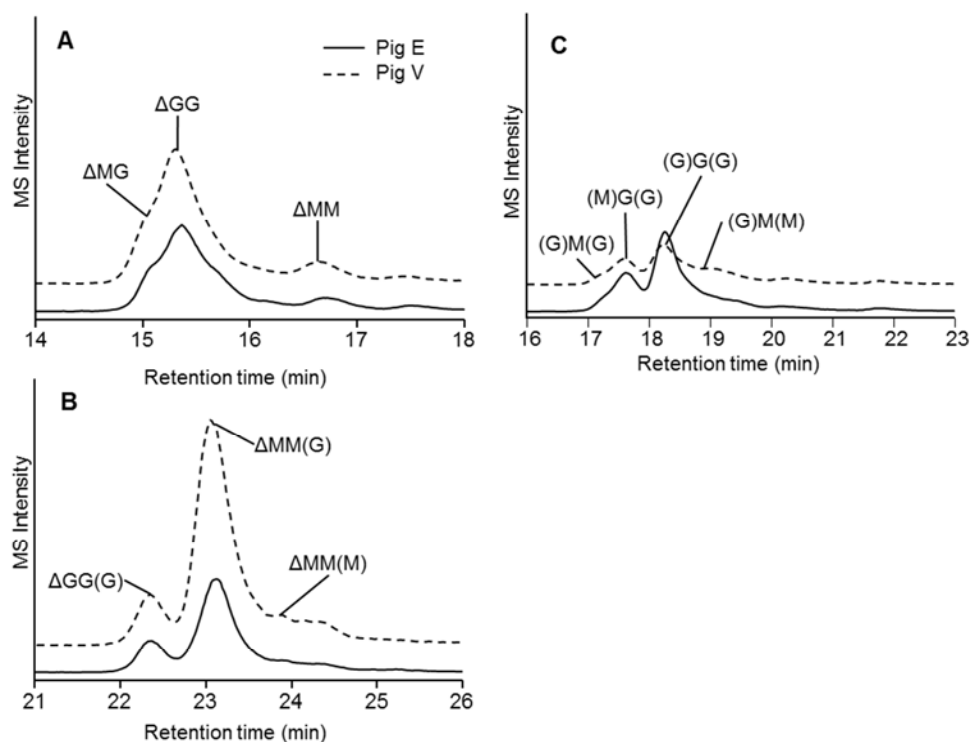


Figure 6–8. Zoomed-in UHPLC-MSⁿ elution patterns of unsaturated alginate oligosaccharides (AOS) trimer (A), unsaturated AOS tetramer (B), and saturated AOS trimer (C) in the faeces of two alginate-fed pigs 7 days after the pigs were fed solely with experimental diets. Δ : 4-deoxy-L-erythro-hex-4-enopyranosyluronate, G: guluronic acid, M: mannuronic acid. The brackets indicate putative annotation of the uronic acid residue. The complete elution pattern of the AOS in the faeces of Pig E can be viewed in Figure 6–5.

6.3.5. Individual variation between pigs in alginate degradation

The AOS composition in the faeces was also used for investigating individual variation between the two pigs used in this study, specifically on alginate degradation in the large intestine. Figure 6–7 shows that the highest proportion of unsaturated AOS was achieved faster for pig V than for pig E. This result seemed to indicate that pig V adapted to alginate more quickly than pig E. This hypothesis was supported by the comparison of the total

AOS content of the samples (Figure 6–6). Although both pigs reached the highest total AOS content on day 7, for pig V it decreased sharply between day 7 and day 14. On the contrary, the total AOS content for pig E was about constant up to day 39. The steep decline of total AOS content for pig V indicated rapid utilisation of AOS, which signifies quick adaptation to alginate.

Table 6–2. Total and soluble alginate contents and apparent digestibility of alginate in the faeces of alginate-fed pigs during a feeding period up to 74 days.

Time (days)	Soluble alginate		Total alginate		Apparent digestibility		
	%(w/w)	G:M ratio	%(w/w)	G:M ratio	G	M	
Pig E							
14	1.8	2.2	21.8	4.5	0.10	0.54	
39	1.7	1.9	23.6	4.8	0.21	0.61	
74	0.5	2.7	17.0	9.4	n.a.	n.a.	
Pig V							
14	0.9	2.0	21.2	4.6	0.16	0.57	
39	0.9	1.6	23.9	4.5	0.24	0.61	
74	0.4	1.9	19.1	6.1	n.a.	n.a.	

G: guluronic acid, M: mannuronic acid, n.a.: apparent digestibility for day 74 could not be calculated because the sample did not contain any indigestible marker.

Individual variation between pigs is also evident from the apparent digestibility data (Table 6–2). On day 14, the digestibility of G for pig E (0.10) was lower than that for pig V (0.16). This again supports the assumption of rapid adaptation of pig V compared to pig E. After adaptation for 39 days, the digestibility of G and M were similar for the two pigs, but the faeces of pig E contained more soluble alginate compared to pig V. On day 74, despite having similar soluble alginate levels, pig E had a lower total alginate content with a higher G:M ratio than that of pig V. The soluble alginate content in the faeces of pig E also had a higher G:M ratio than in the faeces of pig V. With a high G:M ratio for soluble alginate, it was expected that there would be relatively more G-containing AOS in the faeces of pig E on day 74. However, this difference was not observed in the AOS profile due to the very low amounts of AOS with DP 2–4, and the inability to identify isomers larger than DP 4, which were present in higher amounts than the smaller AOS (Figure 6–7). Nevertheless, the lower total alginate content and higher G:M ratio on day 74 suggest that the microbiota in pig E may have a higher ability to solubilise and utilise G than the microbiota in pig V. These results show that the microbiota composition in the large intestine was not influenced

only by diet, but also by other factors. Individual genetic variation may also have an important role, as has been reported before (32).

In summary, the presence of AOS in the faeces of alginate-fed pigs demonstrated that oligosaccharides are present in the large intestine as results of polysaccharide degradation. The ability to identify these oligosaccharides, such as demonstrated in this paper for AOS, is essential to be able to assess the presence of certain oligosaccharides once they are shown to be potentially physiologically active through metabolic processes in the large intestine.

6.4. Supplementary information

Figures for putative annotation of unsaturated tetramer, and saturated trimers are available as supplementary information (Section 6.7).

6.5. Acknowledgements

The authors would like to thank Mark Sanders for the technical assistance in the UHPLC-MSⁿ method development. Carol Souza da Silva, Jing Zhang, Odette Perez and the employees at Nutreco Swine Research Centre are acknowledged for their support in sample collection. This work has been partially funded by the Dutch Ministry of Economic Affairs, Agriculture & Innovation (project KB-05-009-003).

6.6. Supplementary information

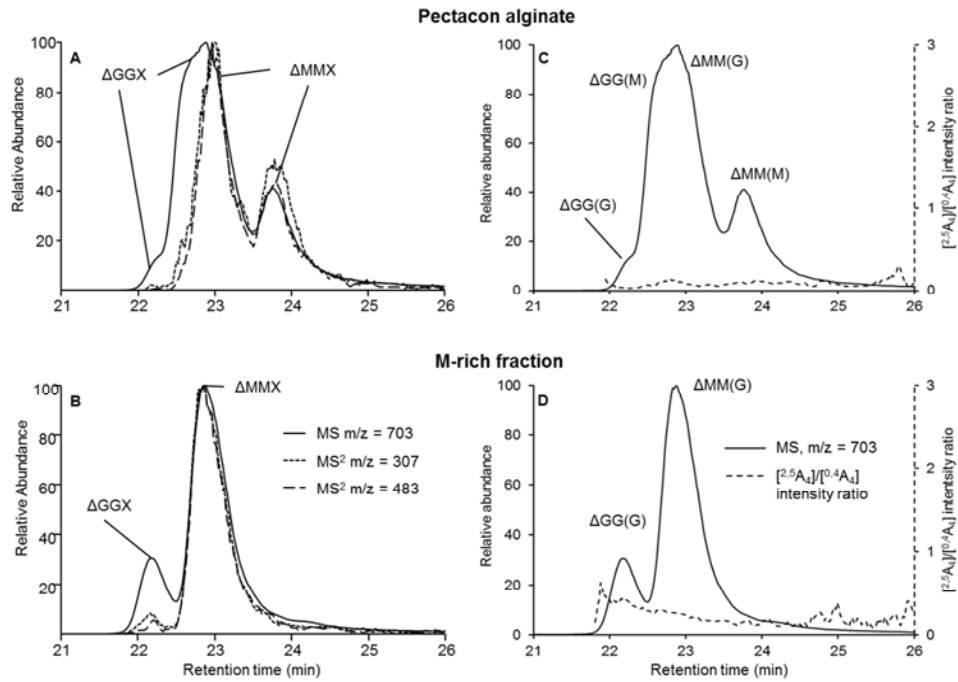


Figure 6-S1. Zoomed-in UHPLC-MSⁿ patterns of unsaturated tetramer isomers from Pectacon alginate and alginate fraction rich in mannuronic acid (M-rich fraction). The internal residues were identified based on the patterns of d-Z₂ (m/z=307) and d-Z₃ (m/z=483) (A,B). The reducing end could not be annotated based on the $[^{2,5}A_4]/[^{0,4}A_4]$ intensity ratio (C,D). Annotation of the reducing end was based on the assumption that the presence of G at the reducing end will decrease the retention time. Brackets indicate putative annotation. Δ : 4-deoxy-L-erythro-hex-4-enopyranosylurionate, G: guluronic acid residue, M: mannuronic acid residue, X: unknown uronic acid residue.

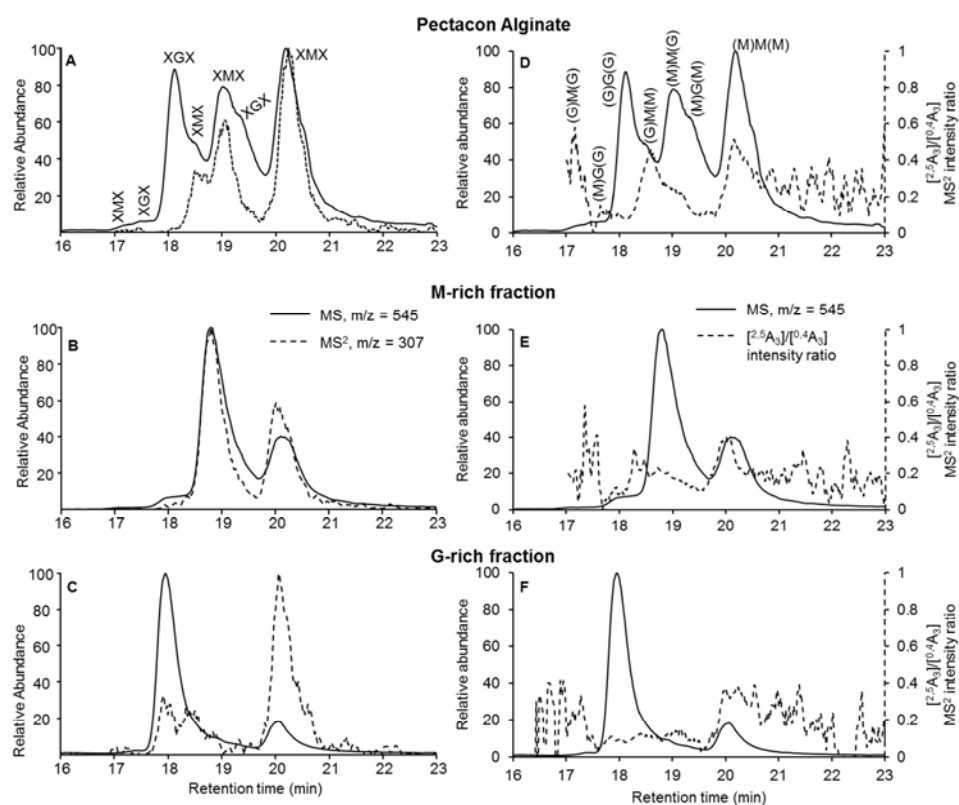


Figure 6–S2. Zoomed-in UHPLC-MSⁿ patterns of saturated trimer isomers from Pectacon alginate, alginate fraction rich in mannuronic acid (M-rich fraction) and alginate fraction rich in guluronic acid (G-rich fraction). The internal residues were identified based on the patterns of d-Z₂ (m/z 307; A–C). The reducing end and the non-reducing end were putatively annotated based on the $[\text{}^{2,5}\text{A}_3]/[\text{}^{0,4}\text{A}_3]$ intensity ratio (D–F) and the presence of the isomer in M-rich and G-rich fractions. Brackets indicate putative annotation. G: guluronic acid residue, M: mannuronic acid residue, X: unknown uronic acid residue.

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Chapter 7

General discussion

7.1. Motivation and aim of the research

The research described in this PhD thesis was part of a larger project entitled 'Food, fibre and health - an integrated approach'. The project aimed to study the role of dietary fibres in inducing satiation and prolonging satiety. As a part of the project, this research was aimed to investigate the changes undergone by different dietary fibres in the gastrointestinal tract (GIT). The approach used was characterisation of the dietary fibres used in *in vitro* and *in vivo* experiments, followed by characterisation of the remaining dietary fibres in the digesta obtained from the experiments. Hence, the degradation of individual dietary fibres and the formation of the degradation products could be monitored. The knowledge gained is expected to provide a basis for further research, which can be focused on the interaction of dietary fibres and its degradation products with the gut microbiota or with the cells of the host, including interactions which may lead to the feeling of satiety.

7.2. Dietary fibres in the upper gastrointestinal tract

Dietary fibres are not degraded in the upper GIT (1). The effects of dietary fibre in the upper GIT are mainly exerted by their physical properties, such as solubility, viscosity, and hydration properties (2). *In vitro*, these properties are often tested on isolated dietary fibres (3, 4) or crude fibre mixtures (5, 6). The physical properties of dietary fibres are determined by their chemical characteristics (2) and particle size (7), and are greatly influenced by various factors, such as thermal (8-10) or physical processing (7) during food preparation and interactions with other components in food (2, 11). Upon consumption, the physical properties of dietary fibres can also be influenced by gastrointestinal conditions (12) as well as by the way of consumption. The same dietary fibre may give different effects when it is supplied in hydrated form in liquid food or in its dehydrated form in capsules (13).

In order to have an estimation of dietary fibre properties in the upper GIT, a simulation of mouth, stomach and small intestinal conditions, which was applied for lupin kernel fibre (5), was adapted for food products. The food products were also used for a human study (Chapter 2). The method includes treatment of the food product by α -amylase to mimic the condition in the mouth, acidification and protein digestion by pepsin to simulate the stomach, followed by neutralisation and digestion by pancreatic enzymes in the presence of bile. It was demonstrated that this relatively simple method could show how the dietary

fibres in cookies could influence the physical conditions in the upper GIT. With a small modification to adjust the volume of the food product, the method was also used for predicting the properties of pectin-containing liquid products in the mouth and in the stomach (13).

The upper GIT simulation described in Chapter 2 is relatively simple. Hence, it can be used for screening for the type and dose of fibres in food products before performing *in vivo* studies related to dietary fibre properties in the upper GIT tract. The results of the simulation were able to partially explain the results of the *in vivo* study. It was found that alginate, which was gelling under the stomach condition, was more satiating than other fibres which were not gelling (Chapter 2). The gelling of alginate in the stomach has been shown *in vivo*, using MRI imaging (12). MRI imaging, however, is costly and cannot be used for screening purposes.

The weak point of the method is that the possible interactions of the dietary fibres with other compounds, such as mucin and calcium, which are secreted by the gastrointestinal glands, were not included in the assessment. This might be the reason why the alginate-containing cookies, which had a relatively low viscosity in the simulated mouth condition, were perceived as sticky by the participants of the *in vivo* study (Chapter 2). This discrepancy might be minimized using a more complex mixture of simulated saliva, gastric juice and small intestinal liquid as developed for analysing the bioavailability of mycotoxins (14).

7.3. Dietary fibres in the large intestine

7.3.1. *In vitro* fermentation

In vitro fermentation is a method, which is often used to predict the fate of dietary fibres in the large intestine and to investigate the products of dietary fibre degradation by the microbiota (15). The results of *in vitro* fermentation of a broad variety of isolated dietary fibres (Chapter 3) showed that dietary fibres were fermented differently by the microbiota from both humans and pigs. The fermentation depended on the constituent monosaccharide and linkage composition, degree of polymerization, and the molecular conformation of the dietary fibres. It was concluded that the fermentation of fibres rich in uronic acids, such as pectin and alginate, results in higher proportions of acetate compared to the fermentation of

fibres rich in neutral constituent monosaccharides, such as guar gum and soy pectin. Although pigs are considered to be one of the most suitable model animals for dietary fibre degradation (16, 17), pig faecal microbiota fermented some fibres very differently compared to human faecal microbiota. For example, cellulose was well fermented by pig faecal microbiota, but it was hardly fermented by human faecal microbiota. On the contrary, the pig faecal microbiota hardly fermented xanthan gum, which was well fermented by human faecal microbiota.

In vitro fermentation of single dietary fibre isolates is useful to unravel how the dietary fibre is fermented by the microbiota and to quantify the fermentation products. However, it does not represent the *in vivo* conditions (15). *In vivo*, dietary fibres are embedded in a complex matrix containing other dietary fibres, other food components, and secreted effluents. Also, in a regular diet, different dietary fibres may be consumed at different times, so that the microbiota is stimulated to adapt to a new source of dietary fibre.

In order to simulate the presence of multiple fibres in the large intestine, an *in vitro* fermentation was performed in which two dietary fibres were supplied simultaneously as well as subsequently to human faecal microbiota (unpublished). As a comparison, the two dietary fibres were also fermented individually. The selected dietary fibres were resistant starch and soy pectin. Resistant starch was selected because it is a commonly consumed dietary fibre during meals (17). The other fibre selected for the study was soy pectin, representing complex pectin as present in fruits and vegetables, with branched molecular structures composed of different monosaccharide residues (18). During *in vitro* fermentation, resistant starch and soy pectin was degraded rapidly by human faecal microbiota (Chapter 3).

The degradation of resistant starch and soy pectin was monitored using HPSEC and HPAEC. Resistant starch was practically insoluble in the fermentation medium and there was no significant solubilisation of high molecular weight starch during *in vitro* fermentation, whereas soy pectin was soluble in the fermentation medium. Therefore, HPSEC was used to monitor the degradation of soy pectin and HPAEC was used to monitor the formation of oligosaccharides from the degradation of resistant starch and soy pectin. The oligosaccharides from resistant starch were identified using maltodextrins as

reference. For soy pectin, although it was not possible to identify the individual oligosaccharides, the HPAEC patterns provided an indication of their presence.

Individual, simultaneous, and sequential *in vitro* fermentation of resistant starch and soy pectin

Materials

Retrograded tapioca starch and soy pectin, as described in Chapter 3.

Inoculum

Faecal microbiota from three human subjects.

Methods

In vitro fermentation was performed as was described (19), with the buffer modified according to Sunvold, et al. (20). For simultaneous *in vitro* fermentation, soy pectin and resistant starch was added at a ratio 1:1 in the beginning of the fermentation. For sequential *in vitro* fermentation, the fermentation was started with the first dietary fibre, and after 8 h of fermentation, the other dietary fibre was added. The fermentation was then continued up to 48 h.

The results of this experiment (Figure 7–1) showed that when microbiota was exposed to a single dietary fibre, it adapted to the fibre and was able to utilise the fibre completely within 48 h (Figure 7–1A,B), shown by the disappearance of peaks in HPSEC patterns for soy pectin, and in HPAEC patterns for resistant starch. The microbiota was also able to adapt to the two fibres simultaneously, shown by the disappearance of the two substrates within 48 h (Figure 7–1C). The simultaneous utilisation of the two dietary fibres substantiates similar results obtained elsewhere for the fermentation of starch and dietary fibres (21).

Adaptation of the microbiota to the changes in dietary fibre sources in the large intestine is analogous to an ecological succession, for which it was stated that the diversity at an adapted condition can be lower than at other stages (22). This statement was substantiated by the experimental results, which showed that when resistant starch and soy pectin were supplied sequentially with an 8 h interval, the utilisation of both substrates after 48 h was lower than that when the substrates were added simultaneously or as single substrates (Figure 7–1D, E). This suggests that during the first 8 h, the microbial diversity decreased due to adaptation to the first substrate.

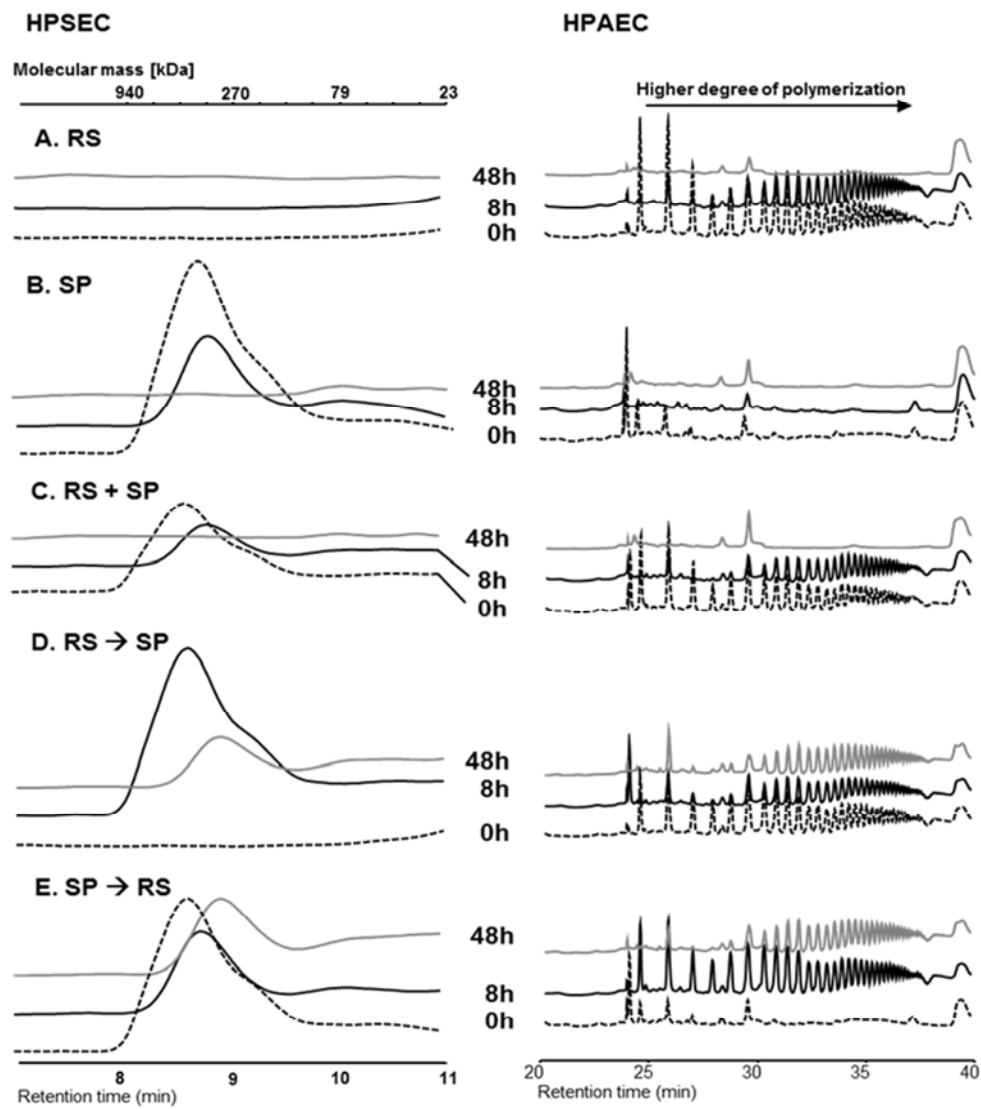


Figure 7-1. HPSEC and HPAEC patterns of fermentation liquid during *in vitro* fermentation of resistant starch (RS) and soy pectin (SP) added to the fermentation medium separately (A, B), simultaneously (C), or sequentially (D, E). The sequential *in vitro* fermentation was performed by adding the second dietary fibre after the first one was fermented for 8 h.

The *in vitro* fermentation results described above demonstrate that the fate of different dietary fibres with different solubilities in the fermentation medium can be monitored individually during fermentation. This individual monitoring of dietary fibres in a mixture

is not possible using the conventional approach, in which the final fermentation products, such as gasses and SCFA, are measured. The approach using HPSEC and HPAEC can also be extended by identifying the remaining oligosaccharides using LC-MS methods. Furthermore, analysis of the constituent monosaccharide composition of the remaining dietary fibres can be conducted if more quantitative results are required, as was exemplified in Chapter 3 for xanthan gum and soy pectin.

7.3.2. *In vivo* studies

The *in vivo* studies described in this thesis were integrated experiments, from which observations and samples were taken and analysed for different aspects: chemical, microbiological, physiological, and behavioural. The latter aspects are parts of other PhD theses belonging to the same overall project. This thesis focused on the chemical aspect, by monitoring the degradation of the various dietary fibres during fermentation in the large intestine.

In this thesis, the degradation of dietary fibres *in vivo* was monitored in various ways. In the study described in Chapter 4, which was designed for another PhD project, an indigestible marker was not included in the diets. Hence, the digestibility was estimated based on polysaccharide degrading enzyme activities as well as on the carbohydrate content and constituent monosaccharide composition of the digesta. Subsequently, another experiment (Exp. 4') was conducted, in which TiO₂ was added into the diets as an indigestible marker. As a result, the apparent digestibility of the dietary fibres could be calculated. The experimental setup of Exp. 4' is described in the box below.

Cross-over pig study (Exp. 4')

This pig study was a cross-over experiment, in which the same pigs received both experimental diets. Growing male pigs were fitted with a cannula at the proximal colon. The basal diet contains 50% of control diet (CON') and 50% of resistant starch diet (RS'). The composition of carbohydrate sources in the experimental diets is presented in Table 7-1. The complete experimental setup and diet composition is described elsewhere (23). The calculation of the digestibility was performed as described in Chapter 5.

In short, after two-weeks fed with the basal diet, one group of pigs (n =5) were fed with CON', and the other group (n =5) was fed with RS'. After 2 weeks, samples were taken from the cannula, and the diet of the pigs was changed to the other diet. After another 2 week period, samples were again taken

Cross-over pig study (Exp. 4', continued)

from the cannula, and the pigs were sacrificed. Digesta were collected from the caecum and 3 different parts of the colon. Samples from 4 pigs were selected to be analysed.

Table 7-1. Carbohydrate composition and indigestible marker (%(w/w)) in the diet for the cross-over pig experiment (Exp. 4').

Ingredient	CON'	RS'
Pregelatinized starch (Paselli WA4, Avebe Food, Veendam, The Netherlands)	35.00	0.00
Retrograded tapioca starch, ~50% resistant starch (C*Actistar 11700, Cargill, Amsterdam, The Netherlands)	0.00	34.26
Wheat	20.00	20.23
Beet pulp	5.00	5.06
Barley	15.00	15.17
TiO ₂	0.20	0.20

In the *in vivo* studies described in Chapter 4 and Exp. 4', the main experimental fibre was resistant starch. In a subsequent experiment (Chapter 5), not only resistant starch, but also alginate was added into the diets. This experiment was focused on monitoring alginate degradation during adaptation. Hence, the feeding time was longer than that in the previous experiments and only faecal samples were taken (Table 7-2). In addition, the alginate excreted in the faeces, including alginate oligosaccharides (AOS), was characterised (Chapter 6).

Table 7-2. Overview presenting main carbohydrate sources, the presence of indigestible marker, feeding time and samples taken during different *in vivo* studies.

Experiment	TiO ₂	Resistant starch	Alginate	Other carbohydrate sources	Feeding time	Samples
Chapter 4	-	+	-	Wheat Barley	2 weeks	Digesta from different parts of the large intestine after slaughter
Exp. 4'	+	+	-	Wheat Barley Sugar beet pulp	2 weeks ¹	Colon digesta and faeces after each treatment, digesta from different parts of the large intestine after slaughter
Chapter 5] Chapter 6]	+	+	+	Wheat Wheat middlings Soybean meal	74 days	Faeces at different time points

-: not present, +: present.

¹ cross-over experimental design, 2 weeks per diet.

Table 7–2 shows that in the three pig studies, the pig diets also contained dietary fibres from other carbohydrate sources than the added dietary fibres (Table 7–2). These additional carbohydrate sources contain NSPs. In the next sections, the degradation of resistant starch, alginate and other NSPs are discussed and compared with the results obtained from *in vitro* fermentation.

7.3.2.1. Resistant starch

In vivo, resistant starch was rapidly fermented in the caecum, as was shown by the low amount of starch in the proximal colon (Chapter 4). This was supported by the results of the cross-over pig study (Exp. 4'; Figure 7–2), in which the digestibility of starch at the caecum and at the proximal colon was higher than the estimated value if resistant starch had not been utilised (~0.66).

The immediate utilisation of resistant starch by the microbiota in the caecum is not reflected in the *in vitro* fermentation results of the same resistant starch by pig faecal microbiota (Chapter 3). This discrepancy could be caused by several factors. First, the pig faecal microbiota used for *in vitro* fermentation (Chapter 3) might not be adapted to resistant starch. Second, the composition of pig faecal microbiota might be different from pig caecal microbiota (24). Third, the resistant starch supplied to the microbiota in the *in vitro* fermentation still contained some digestible starch. This condition does not represent the *in vivo* condition, in which only the resistant starch will reach the large intestine.

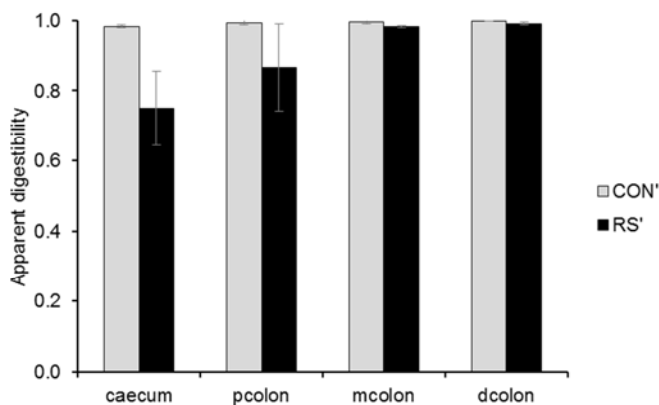


Figure 7-2. Apparent digestibility of starch at different parts of the large intestine of pigs fed with a control diet (CON') and pigs fed with resistant starch rich diet (RS') after 2 weeks of feeding (Exp. 4'). pcolon: proximal colon, mcolon: middle colon, dcolon: distal colon.

The pig caecal microbiota needed less than two weeks to adapt to resistant starch. This was shown by the utilisation of resistant starch in the caecum after only 2 weeks of feeding with RS-containing diet (Chapter 4, Exp. 4'). The adaptation of the microbiota to resistant starch may include a change in microbial composition and stimulation of the production of the enzymes necessary for the utilisation of the dietary fibre (25, 26). The latter was confirmed by the increase of α -amylase and amyloglucosidase enzyme activities, especially at the proximal parts of the large intestine (Chapter 4).

It was also shown that the presence of resistant starch in the diet delayed the degradation of NSPs (Chapter 4). This effect of resistant starch on NSPs will be discussed in more detail in Section 7.3.2.3. The effects of resistant starch described in this thesis, however, might be specific for retrograded tapioca starch. It has been reported that different types of resistant starch (27), as well as different forms of retrograded starch (28) have different effects on human microbiota composition and the resulting SCFA composition.

7.3.2.2. Non-starch polysaccharides (NSPs)

As stated before, besides resistant starch, the pig diets in Chapter 4 and in Exp. 4' contained carbohydrates from other ingredients (Table 7–2). These carbohydrate sources contain NSPs with different compositions (Table 7–3). Except mixed-linkage β -glucan, most of the dietary fibres in these ingredients are insoluble in water.

Table 7–3. Constituent monosaccharide composition of non-starch polysaccharides in the carbohydrate sources and in the diets used in *in vivo* pig studies.

Ingredient	monosaccharide composition ¹ (mol%)								Main dietary fibres	Ref
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA		
Wheat	t	t	31	45	t	2	21	1	arabinoxylan mixed-linkage β -glucans cellulose	(29)
Barley	t	t	21	32	2	1	43	1	mixed-linkage β -glucans arabinoxylan cellulose	(29)
Sugar beet pulp	2	n.a.	31	3	2	6	31	25	pectins cellulose arabinan	(30)

t: detected in trace amounts, n.a. : not mentioned in the reference.

¹ calculated based on the %(w/w) as presented in the references.

The NSP constituent monosaccharide composition of the diets can then be used as a means to calculate the digestibility of dietary fibres, if an indigestible marker is included in the diet. Results from Exp. 4⁷ (Figure 7–3) demonstrated that the digestibility of NSPs in the colon was lower for RS⁷-fed pigs than for CON⁷-fed pigs. The digestibility of NSPs in the faeces was also different between diets, but the difference was less than that in the colon, suggesting a delayed degradation of NSPs in the presence of resistant starch. Hence, the conclusion drawn in Chapter 4 based on the carbohydrate content, constituent monosaccharide composition and activities of polysaccharide degrading enzymes are confirmed.

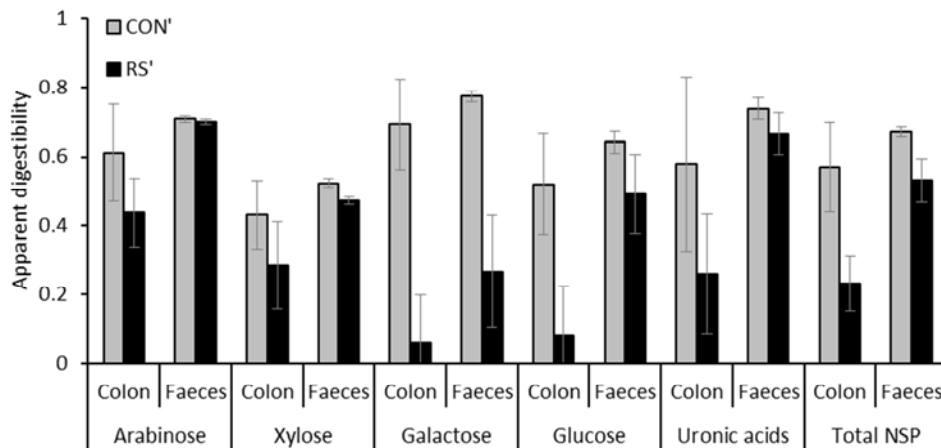


Figure 7-3. Apparent digestibility of non-starch polysaccharide (NSP) constituent monosaccharides in the proximal colon and in the faeces of cannulated pigs after feeding with control diet (CON⁷) or resistant starch rich diet (RS⁷) in Exp. 4⁷.

The delayed utilisation of NSPs in the presence of resistant starch is not in accordance with the simultaneous *in vitro* fermentation of resistant starch and soy pectin (Section 7.3.1). In addition to the reasons given in the discussion of the difference between *in vitro* and *in vivo* resistant starch degradation (Section 7.3.2.1), this discrepancy can be caused by the different NSPs used. It might also be caused by the different ratios of resistant starch to NSP in the *in vitro* fermentation as compared to the ratio in the *in vivo* pig study.

Figure 7–3 illustrates the digestibility of individual NSP constituent monosaccharides. The lower digestibility of NSPs with the inclusion of resistant starch in the diet seems to be most prominent for galactose and glucose, both in the colon and in the faeces. These

monosaccharides are also present in microbial cells in human faeces (31, 32) and those in the rumen of sheep (33). In addition, resistant starch was reported to be able to increase microbial mass in the large intestine (34). Therefore, the digestibility of glucose- and galactose might be underestimated.

In Exp. 4', the main fibres in the NSPs included arabinoxylan, sugar beet pectin, mixed-linkage β -glucans, and cellulose. The digestibility of arabinoxylan could only be estimated from the digestibility of xylose because arabinose is also present in sugar beet pectin. Figure 7-3 shows that the digestibility of xylose tended to be lower than that of arabinose. Most of the arabinoxylan in the diet was insoluble in water. It was expected that the digestibility of xylose would be higher than that of arabinose because for insoluble arabinoxylan, it has been reported that the digestibility of highly substituted arabinoxylan was lower than that of less substituted arabinoxylan (35). Hence, the higher digestibility of arabinose compared to xylose in Exp. 4' may indicate that arabinose from sugar beet pectin was utilised more easily by the microbiota than arabinose from wheat arabinoxylan.

Using a similar approach, the digestibility of sugar beet pectin can be roughly estimated from the digestibility of uronic acid. However, uronic acids are also present in the arabinoxylan from wheat and barley. In general, in the presence of resistant starch, the uronic acid digestibility in the colon was lowered more than the digestibility of arabinose and xylose. This may show that the microbiota which is responsible for uronic acid utilisation is different from those responsible for the utilisation of arabinose or xylose. However, in the faeces, the difference between diets disappeared, which may show that after the resistant starch was depleted, the microbiota which is able to utilise uronic acids was able to grow and compete with the other microbiota.

Although not measured separately in Exp. 4', the digestibility of mixed-linkage β -glucans can be assumed to be near to 1.0, based on results from the previous experiment (Chapter 4) and available literature (36). Mixed-linkage β -glucans was also fermented rapidly during *in vitro* fermentation (Chapter 3). The digestibility of cellulose can be estimated from the digestibility of insoluble NSP glucose (0.58 ± 0.07 and 0.45 ± 0.12 for faecal samples from Control and RS, respectively). These values are higher than the digestibility of cellulose from wheat bran (0.30; (36)) and are similar to the digestibility of cellulose from barley (0.56; (37)).

7.3.2.3. Alginate

Alginate was selected for the *in vivo* pig study described in Chapter 5 because it was shown to be more satiating than other polysaccharides, such as guar gum and cellulose (Chapter 2). Alginate is composed of guluronic acid (G) and mannuronic acid (M), which are arranged in a linear molecule. The alginate used in the pig study (Chapter 5) was rich in G, similar to the one used in the *in vivo* human study (Chapter 2).

In vitro, alginate was fermented slowly by faecal microbiota from humans and pigs (Chapter 3). *In vivo*, it was shown that more than 40 %(w/w) of the alginate consumed by the pigs was excreted in the faeces (Chapter 5). Also, the microbiota needed a long time to be adapted to alginate (>39 days). The slow adaptation of the microbiota to alginate, as well as the slow fermentation of alginate *in vitro* could be explained by the time needed for the stimulation of the microbiota capable of producing alginate-degrading enzymes, as shown in Table 7-4. The table presents results of another unpublished *in vitro* fermentation, in which four substrates were fermented by human faecal microflora. The substrates were high methylated (HM) pectin, guar gum, soy pectin, and alginate. These substrates were selected because of their solubility in the fermentation medium and their diverse constituent monosaccharide composition and molecular structure. The fermentation medium was formulated as described earlier (Section 7.3.1).

It was hypothesized that after fermentation, the fermentation liquid contains polysaccharide degrading enzymes with an optimal composition to degrade the substrates. Therefore, the cell-free fermentation liquids were tested for polysaccharide degrading enzyme activities. The incubation of the enzyme-substrate mixture was performed at 37 °C for 24 h. The ability of the enzymes to degrade the substrates was then monitored using HPSEC and compared with each other. The activity of the enzymes were then classified based on the extent of substrate degradation as shown by the shift in the retention times in the HPSEC patterns.

Table 7-4 shows that alginate degrading enzymes were present only in the fermentation liquid of alginate. This result shows that the growth of the microbiota able to produce alginate degrading enzymes had to be stimulated or that the production of alginate-degrading enzymes in the microbiota had to be induced. This result demonstrates that the microbiota composition as well as the metabolic pathways of some bacteria might be

altered as a result of dietary fibre fermentation, in accordance with the results described elsewhere (38).

Table 7-4. Polysaccharide degrading activities found in fermentation liquid obtained by *in vitro* fermentation of alginate, pectin, soy pectin and guar gum.

Fermentation liquid from the fermentation of:	Enzyme activity towards:					
	Alginate	Pectin	Soy pectin	Guar gum	Galactan	Arabinan
Alginate	++	+	++	++	+	+
HM Pectin	-	++	+++	+++	++	++
Soy pectin	-	+	+++	++	++	+
Guar gum	-	-	+	++	+	-

-: no activity, +: little activity, ++: moderate activity, +++: high activity.

The alginate degrading enzymes in the *in vitro* fermentation described above were not investigated further. However, from the *in vivo* study (Chapter 5, 6), it was shown that the alginate degrading enzymes in the large intestine of pigs were mainly alginate lyases. The results strongly indicate that mannuronate lyase was present, because the digestibility of M was higher than that of G. Guluronate lyase could also be present, as indicated by the presence of alginate oligosaccharides (AOS) containing only guluronates. The digestibility of G, however, was low, resulting in a high G:M ratio in the indigestible alginate. The indigestible alginate was mostly insoluble, probably because of interaction with calcium or other compounds such as polyphenols and proteins. Interactions of alginate with calcium (39) and polyphenols (40) have been shown to inhibit alginate degradation by enzymes.

The higher utilisation of M compared to that of G leads to the hypothesis that if the high G alginate in the diet is replaced by M-rich alginate, the outcome of the study could be different. The digestibility of M-rich alginate can be higher than that of G-rich alginate. As a consequence, alginate degradation might take place in more proximal parts of the large intestine. There can also be variations in alginate degradation depending on the distribution of the uronic acid residues. Due to these possible variations, the chemical properties of alginate, including the composition of the uronic acids, should be described whenever an alginate is used in a study.

7.3.2.4. Fibre degradation products in the large intestine

During utilization of dietary fibres, some microbiota produces extracellular enzymes to degrade polysaccharides, yielding oligosaccharides and monosaccharides (41). The oligosaccharides produced may have other physiological effects than the polysaccharides. For example, the oligosaccharides can have a prebiotic activity whereas the polymer does not have such an activity, through a cross-feeding mechanism (41). Oligosaccharides, including those which are not prebiotic, can also be physiologically active through contact with mammalian cells, such as immune cells and endothelial cells. The effects may include modification of the immune system and anti-tumor activity (42). Interactions between oligosaccharides and the cells, as mediated by cell receptors, can be highly specific depending on the molecular structure and the conformation of the oligosaccharides (42). Therefore, it was attempted to identify oligosaccharides from the degradation of dietary fibres by the microbiota in the large intestine of pigs.

Oligomeric degradation products from non-starch polysaccharides (NSPs)

The first attempt to identify oligosaccharides in the digesta was performed on the samples from Exp. 4', in which pigs were fed with resistant starch and NSPs from wheat, barley and sugar beet pulp. The methods used were HPAEC and UHPLC-MSⁿ. Only hexose oligosaccharides, which were mostly maltodextrins, were detected in the samples. The difficulty in the detection and identification of oligosaccharides in the digesta might be explained by the low amounts of soluble carbohydrates present in the samples. The NSPs in the diets for Exp 4' was mostly insoluble. It has been reported that some bacteria have the ability to attach to the surface of insoluble substrates and they have polysaccharide degrading enzymes attached to their cell wall (41). In this case the monosaccharides and oligosaccharides produced from the degradation can be transported into the cell immediately for further utilisation. As a result, there may be no accumulation of oligosaccharides in the digesta.

Alginate oligosaccharides (AOS)

In the study with alginate-containing diets (Chapter 5, 6), AOS were detected in the faeces, although the alginate level in the diet (5 %(w/w)) was in the same order of magnitude as the NSP level in Exp. 4' (7.7 %(w/w)). The higher level of AOS in the faeces compared to the level of oligosaccharides from other NSPs in Exp. 4' described above shows that the

microbiota utilised AOS less rapidly than it utilised oligosaccharides from other NSPs in Exp. 4'. The presence AOS in the faeces also indicates that AOS were also present in the large intestine. Saturated and unsaturated AOS were present, and some specific AOS in the faeces were successfully identified using UHPLC-MSⁿ (Chapter 6). The utilization of the AOS was shown to be affected by the presence of a G residue at the reducing end, and the microbiota preferred to utilise AOS that are composed of solely M residues over other AOS. After 74 days, however, the the microbiota adapted to the alginate and became less selective in AOS utilisation, as shown by the low amount of soluble alginate degradation products in the sample.

7.3.2.5. Individual variation in *in vivo* studies

Within the *in vivo* studies (Chapter 4-6) it was evident that variation between animals has an important role in fibre degradation in the large intestine. The individual variation includes the rate of fibre degradation and the adaptation time needed for a new fibre. The variations may result in different sites of fermentation of a dietary fibre in the large intestine of different individuals. In addition, the fermentation pathways might also be different; resulting in formation of different oligosaccharides from the same parental polysaccharides. As a consequence, the individual variation may therefore cause different responses of the animal towards the same dietary fibre. All of these individual variations can be attributed to the microbial composition in the large intestine, which is influenced by environmental as well as by host genetic factor (43, 44).

7.4. Future perspectives

7.4.1. Fibre classification

The studies described in this thesis substantiated the notion that dietary fibres should be classified based on their chemical structures rather than on their physical or physiological properties, such as solubility in water or fermentability. Solubility in water does not always reflect solubility in the GIT (Chapter 2, 5). The solubility of dietary fibres in water also depends on specific molecular structure and interaction with other compounds, such as calcium, proteins, or polyphenols. Classification of dietary fibres based on their fermentability is also ambiguous. As an example, for alginate, the digestibility of M was

higher than that of G (Chapter 5). Hence, it is hypothesized that an M-rich alginate would be more fermentable than a G-rich alginate.

7.4.2. Dietary fibre research

The diversity of dietary fibres suggests that in future studies using dietary fibres, either *in vitro* or *in vivo*, the information about the fibre should include detailed information on the chemical characteristics of the fibre. The processing conditions and the presence of other components, which may interact with the dietary fibres, have to be considered as well. It has been pointed out that the lack of information about the chemical characteristics of the dietary fibre may complicate comparisons of experimental data and may even lead to contradicting information for the same type of fibre (45, 46).

Besides the chemical characteristics of the fibres, the adaptation time to the fibres has to be considered carefully during an *in vivo* study. Adaptation to the dietary fibre may include changes in microbiota composition or in microbial enzyme expression (38) and changes in intestinal morphology (47). Due to these changes, an effect that is observed after a short time of feeding may disappear after continuous feeding of the same fibre (Chapter 5). For *in vitro* fermentation, the adaptation state of the faecal microbiota to the fibre of interest should also be considered, mainly by assessing the diets of the donors before the sampling for faecal microbiota. Microbiota that is adapted to a certain fibre can also be obtained by performing a pre-fermentation, in which the inoculum is grown on the selected fibre before it is used for the main *in vitro* fermentation.

Another factor that has to be taken into account is individual variation between subjects, either animals or humans, in *in vivo* studies or faecal microbiota donors for *in vitro* fermentations. The individual variation may cause a fibre to give the desired effects to an individual, whereas it does not have any effect on other individuals.

7.4.3. Monitoring degradation of individual dietary fibres

This thesis demonstrated that monitoring the degradation of individual dietary fibres in *in vitro* and *in vivo* studies is possible with the help of separation techniques, such as HPSEC for molecular mass, HPAEC for monosaccharide and oligosaccharide profiling, and UHPLC-MSⁿ for identification of oligosaccharides. Nevertheless, these techniques can only be applied for dietary fibres and degradation products that are soluble in water.

Insoluble dietary fibres cannot be characterised using these methods. Nevertheless, it was demonstrated in Section 7.3.2.3 that the degradation of insoluble dietary fibres can be monitored by carefully analysing the digestibility of individual constituent monosaccharides. More detailed results for insoluble dietary fibres can be obtained by following the extraction methods using chelating agents and alkali that are often used for characterisation of plant cell wall materials (48).

Another issue that needs to be considered in monitoring the degradation of dietary fibres by microbiota is the presence of microbial cells. These cells also contain carbohydrates, which may interfere with the analyses of the constituent monosaccharide composition of dietary fibres, as shown in Section 7.3.2.3. Methods to fractionate digesta into soluble compounds, plant-derived compounds and bacterial cells are available (31, 32, 49), but these methods are tedious and not applicable for high throughput analyses.

7.4.4. Production of dietary fibres: the potential of oligosaccharides

Most of dietary fibres are initially produced to be used as thickeners, stabilisers, or bulking agents in food. The same polysaccharides may also be added into food as dietary fibres: an ingredient with health functionality. In addition to the polysaccharides, oligosaccharides that are not digested or absorbed in the small intestine are recently also included as dietary fibres. These oligosaccharides, although lacking the gelling ability and viscosity, can also be physiologically active by having a prebiotic activity (50, 51). Specific oligosaccharides can also interact with immune or endothelial cells (42).

The production of specific oligosaccharides or mixtures enriched with specific oligosaccharides will probably be costly. Moreover, upon consumption, the oligosaccharides might be rapidly utilised by the microbiota before they reach the targeted site in the colon. As an alternative, oligosaccharides can be produced from polysaccharides by the microbiota present in the large intestine (Chapter 6). Nevertheless, the 'on-site' production of specific oligosaccharides largely depends on the microbiota composition of the individuals. In order to obtain the desired specific oligosaccharide for every individual, the synbiotic concept in which living microbiota and the substrate are delivered simultaneously (52) can be explored. To ensure that the microbiota and the substrate reach the targeted site in the colon, specific colon delivery systems as known for drug administration delivery (53, 54), can be considered.

7.4.5. Consumption of dietary fibres

The results of this thesis show that the diet should contain sufficient amounts of dietary fibres to ensure fermentation occurs in the entire large intestine. The current recommendation for dietary fibre consumption is 25 g a day (55). This recommendation does not take into account the diversity of dietary fibres and does not distinguish between dietary fibres in native form or isolated dietary fibres in supplements.

Consumption of diverse dietary fibres with different fermentabilities is also important to ensure that fermentation occurs in the entire large intestine. The results of this thesis suggest that consumption of the same dietary fibre for a long period might reduce the microbial diversity and lower the capability of the microbiota to quickly adapt to another fibre. In addition, if the microbiota is highly adapted to a certain dietary fibre, the fibre may be fermented very rapidly at the proximal large intestine, which may result in a lack of fermentable carbohydrates at the distal parts of the colon. Hence, the composition of the diet should not be constant over time.

A broad diversity of dietary fibres can easily be obtained from the consumption of edible plants, such as fruits, vegetables and cereals. Consuming natural sources of dietary fibres may also have additional advantage because other compounds that are beneficial for health are often associated with the dietary fibre in the plant cell wall. For example, it has been reported that alginate-containing seaweed extract may inhibit the activity of α -amylase. This may reduce the glycemic index of carbohydrate-rich food. On the contrary, isolated alginate did not give similar effect (56).

7.5. Concluding remarks

Degradation of dietary fibres in the large intestine is usually monitored by establishing the apparent digestibility and measuring fermentation end-products. The results of this study demonstrated that the conventional approach can be complemented with monitoring of the polysaccharide degrading enzyme activities and dietary fibre degradation products in the digesta. Various separation techniques were shown to be useful for monitoring the degradation of individual dietary fibres, especially if the fibres and their degradation products are soluble in water.

It was shown that different dietary fibres exhibited different physical characteristics in the upper GIT and the physical characteristics of one fibre can change between the different parts of the GIT. In the large intestine, diverse dietary fibres are fermented differently by the microbiota, which may affect the health effects of the dietary fibres. In addition, it was demonstrated that the large intestinal microbiota needed different adaptation times for the different fibres they encountered.

This study also raises the awareness that oligosaccharides can be present in the large intestine as a result of NSP degradation. These oligosaccharides may have a role in the mechanism by which dietary fibres are physiologically beneficial. However, the bio-functionality of these oligosaccharides *in vivo* has yet to be revealed. Further research aiming to unravel the working mechanisms of dietary fibres, therefore, should consider the presence of these oligosaccharides and their possible biofunctionalities. In addition, the location of oligosaccharide formation, the type of microbiota involved and the factors influencing the formation of specific oligosaccharides in the large intestine still need to be investigated.

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Summary

Samenvatting

Summary

Consumption of dietary fibres is thought to be beneficial for health. Dietary fibres are very diverse in chemical structure and hence they may have diverse biofunctionalities. In **Chapter 1** it is described that dietary fibres in the upper gastrointestinal tract (GIT) mainly function through their physical properties, such as viscosity and water holding capacity. In the large intestine, part of the functionality of dietary fibres is mediated through the fibre fermentation by the gut microbiota. Many *in vitro* and *in vivo* studies have been performed in relation to the effects of dietary fibres in the upper GIT and the fermentation of dietary fibres in the large intestine. However, the dietary fibres and their degradation products in the GIT were not often monitored in detail. Hence, the aim of this study was to investigate the fate of dietary fibres in the gastrointestinal tract (GIT), especially in the large intestine where most of the degradation of dietary fibres occurs.

First, the effects of dietary fibres with different physical properties (cellulose, guar gum and alginate) to the induction of satiation in humans are described in **Chapter 2**. The use of a simple *in vitro* simulation of the upper GIT for analysing the physical properties of dietary fibre in food is also described. It was shown that alginate is more satiating than cellulose or guar gum. The satiating effect of alginate is partially explained by the ability of alginate to form a gel under the acidic condition in the stomach, as shown by the *in vitro* upper GIT simulation.

The investigation of the fate of dietary fibre in the GIT was continued with an *in vitro* fermentation of a number of dietary fibres having different chemical characteristics, as described in **Chapter 3**. Faecal microbiota from humans and pigs were used as inoculum. It was concluded that dietary fibres are fermented differently depending on their constituent monosaccharide and linkage compositions, degree of polymerization and molecular conformation of the polysaccharides, yielding different amounts of gasses and short chain fatty acids. It was also shown that cellulose was hardly fermented by human faecal microbiota, but was fermented by the pig faecal microbiota. On the other hand, human faecal microbiota had more ability to ferment xanthan gum than the pig faecal microbiota.

In **Chapter 4**, the effects of resistant starch, a dietary fibre that was found to be satiating, on the degradation of non-starch polysaccharides (NSPs) in the large intestine of pigs are described. The NSPs were mainly cellulose, arabinoxylan and mixed-linkage β -glucans

from wheat and barley. Resistant starch, which was rapidly utilised by the microbiota in the caecum, was shown to delay the degradation of the arabinoxylan and mixed-linkage β -glucans in the large intestine. In this experiment, indigestible marker was not available for quantification of the digestibility of the dietary fibres. Therefore, the conclusions were drawn based on the constituent monosaccharides and content of NSPs, as well as the polysaccharide degrading enzyme activities in the digesta from the large intestine. These conclusions were further supported by the results of an additional experiment in which TiO_2 was added as an indigestible marker to the diets, as described in the General Discussion section.

The *in vivo* pig study was followed up by another study, which included alginate in the diet, besides resistant starch and other NSPs from the other carbohydrate sources, as described in **Chapter 5**. Alginate is a linear polysaccharide from seaweed, composed of guluronic acid (G) and mannuronic acid (M). This study was focused on the alginate degradation in pigs during a feeding period up to 74 days. Results of this study show that the G-rich alginate that was used in this study was only partly utilised by the microbiota in pigs, with more than 40 % (w/w) of the alginate intake excreted in the faeces. This resistant alginate was enriched in G, showing that the microbiota prefers to utilise M rather than G. It was also shown that the microbiota in the large intestine of the pigs needed more than 39 days to be adapted to the alginate.

In **Chapter 6**, the alginate oligosaccharides (AOS) in pig faeces were analysed using UHPLC-MSⁿ. Both unsaturated and saturated AOS were present in the faeces of the alginate-fed pigs. The presence and the increasing amounts of unsaturated AOS during the adaptation period indicate that the microbiota produced alginate lyases. Various isomers of AOS trimers and tetramers were detected and identified. In combination with the results from Chapter 5, it was concluded that the presence of G at the reducing end of AOS affects the AOS utilisation by the microbiota that was not adapted to alginate. After adaptation, the microbiota was able to utilise a broader range of AOS. In this chapter, individual variation between pigs is also addressed, with the conclusion that the microbiota in one pig had a different ability to utilise G-rich alginate compared to the microbiota in the other pig.

In **Chapter 7**, the *in vitro* results related to the fate of dietary fibres in the GIT are discussed in relation to the results of the *in vivo* studies. The degradation of resistant starch,

alginate and other NSPs in the large intestine are addressed separately. This is done by combining results obtained from the experiments as described in previous chapters as well as results from additional experiments. The additional experiments include a pig study in which the digestibility of starch and dietary fibres could be calculated. Results of this additional pig study support the conclusions drawn in Chapter 4. An individual, simultaneous, and sequential *in vitro* fermentation of resistant starch and soy pectin is described as well, which gives an insight that intestinal microbiota that is adapted to a single source of dietary fibre may have a reduced adaptability to other fibres. Finally, the possible impact of this study to dietary fibre research, production, and consumption is also discussed.

Samenvatting

Het consumeren van voedingsvezels wordt gunstig geacht voor de gezondheid. Voedingsvezels zijn qua chemische structuur zeer divers en kunnen daardoor diverse biofunctionaliteiten bezitten. In **Hoofdstuk 1** wordt beschreven dat voedingsvezels in het bovenste deel van het maag-darmkanaal vooral functioneren middels hun fysische eigenschappen als viscositeit en water bindend vermogen. In de dikke darm wordt de functionaliteit van voedingsvezels deels bewerkstelligd door vezelfermentatie door darmbacteriën. Vele *in vitro* en *in vivo* studies zijn uitgevoerd om de effecten van voedingsvezels op het bovenste deel van het maag-darmkanaal en fermentatie in de dikke darm te onderzoeken. De voedingsvezels zelf en hun afbraakproducten in het maag-darmkanaal worden echter niet vaak in detail onderzocht. Daarom is het doel van deze studie het onderzoeken van het lot van voedingsvezels in het maag-darmkanaal, met name in de dikke darm waar de meeste afbraak van voedingsvezels plaatsvindt.

Allereerst worden de effecten van voedingsvezels met verschillende fysische eigenschappen (cellulose, guar gum en algiinaat) op het induceren van verzadiging in mensen beschreven in **Hoofdstuk 2**. Ook het gebruik van een simpele *in vitro* simulatie van de bovenste delen van het maag-darmkanaal voor het analyseren van fysische eigenschappen van voedingsvezel wordt beschreven. Het verzadigende effect van algiinaat kan deels worden verklaard doordat algiinaat een gel kan vormen onder de zure omstandigheden in de maag.

Het onderzoeken van het lot van voedingsvezels in het maag-darmkanaal werd vervolgd met een *in vitro* fermentatie van een aantal voedingsvezels met verschillende chemische kenmerken, zoals beschreven in **Hoofdstuk 3**. Fecale microbiota van mensen en varkens werden als inoculum gebruikt. De conclusie is dat voedingsvezels op verschillende wijze gefermenteerd worden, afhankelijk van hun monosaccharide- en bindingstype samenstelling, polymerisatie graad en moleculaire conformatie van de polysaccharide, waardoor ook verschillende hoeveelheden gassen en kortketenige vetzuren geproduceerd werden. Ook werd aangetoond dat cellulose nauwelijks gefermenteerd wordt door menselijk microbiota, maar wel gefermenteerd wordt door de fecale microbiota van varkens. Aan de andere kant zijn menselijke fecale microbiota beter in staat om xanthaan gum te fermenteren dan de fecale microbiota van varkens.

In **Hoofdstuk 4** worden de effecten van resistent zetmeel op de afbreekbaarheid van niet-zetmeel polysacchariden in de dikke darm van varkens beschreven. Resistent zetmeel is een voedingsvezel die een verzadigend effect lijkt te hebben. Aanwezige niet-zetmeel polysacchariden waren voornamelijk cellulose, arabinoxylaan en β -glucanen uit tarwe en gerst. Aangetoond werd dat resistent zetmeel, dat snel verbruikt wordt door de microbiota in de blinde darm, de afbraak van arabinoxylanen en β -glucanen in de dikke darm vertraagd. In dit experiment was de onverteerbare markerstof niet beschikbaar voor het kwantificeren van de verteerbaarheid van voedingsvezels. Daardoor zijn de conclusies zowel gebaseerd op monosaccharide samenstelling en gehalte van niet-zetmeel polysacchariden, en op de activiteit van polysacchariden afbrekende enzymen in de digesta uit de dikke darm. Deze conclusies worden onderbouwd door een extra experiment waarin titanium oxide werd toegevoegd als een onverteerbare markerstof in het voer, zoals beschreven in de algemene discussie.

De *in vivo* studie in varkens werd vervolgd met een andere studie, waarbij ook algiinaat aan het voer werd toegevoegd, naast resistent zetmeel en andere niet-zetmeel polysacchariden uit andere bronnen, zoals beschreven in **Hoofdstuk 5**. Algiinaat is een lineaire polysaccharide uit zeewier, bestaande uit guluronzuur (G) en mannuronzuur (M). De focus van deze studie was de afbreekbaarheid van algiinaat in varkens gedurende een voerperiode van 74 dagen. Resultaten uit deze studie laten zien dat het G-rijke algiinaat, die in deze studie gebruikt is, slechts deels verbruikt kon worden door de microbiota in varkens, waarbij meer dan 40 % van het opgenomen algiinaat uitgescheiden werd in de feces. Dit resistente algiinaat was verrijkt in G, wat laat zien dat microbiota het gebruik van M verkiezen boven het gebruik van G. Aangetoond werd dat microbiota in de dikke darm van varkens meer dan 39 dagen nodig heeft om zich aan te passen aan het algiinaat dieet.

In **Hoofdstuk 6** werden de algiinaat oligosacchariden (AOS) in varkensfeces met UHPLC-MSⁿ geanalyseerd. Zowel onverzadigde als verzadigde AOS waren aanwezig in de feces van varkens die algiinaat gevoerd kregen. De aanwezigheid van en toename in onverzadigde AOS gedurende de aanpassingsfase duiden erop dat de microbiota algiinaat lyase enzymen produceren. Verschillende isomeren van AOS trimeren en tetrameren werden geïdentificeerd. In combinatie met de resultaten uit Hoofdstuk 5 kon worden geconcludeerd dat de aanwezigheid van G aan het reducerende uiteinde van de AOS het gebruik van AOS door de niet aangepaste microbiota beïnvloed. Na aanpassing is de microbiota in staat om

een breder scala aan AOS te gebruiken. In dit hoofdstuk wordt de individuele variatie tussen varkens besproken, met als conclusie dat de microbiota in het ene varken op een andere manier G-rijk alginaat gebruikten vergeleken met de microbiota in een ander varken.

In **Hoofdstuk 7** worden de resultaten van *in vitro* studies naar het effect van voedingsvezels in het maag-darmkanaal gerelateerd aan de resultaten van de *in vivo* studies. De afbraak van resistent zetmeel, alginaat en andere niet-zetmeel polysacchariden in de dikke darm worden apart besproken, en in combinatie met resultaten die verkregen zijn in extra experimenten. Deze extra experimenten omvatten ook een studie met varkens waarbij de verteerbaarheid van zetmeel en voedingsvezels kon worden berekend. De resultaten ondersteunen de conclusies uit Hoofdstuk 4. Een individuele, simultane of opeenvolgende *in vitro* fermentatie van resistent zetmeel en soya pectine wordt beschreven en geeft inzicht in het feit dat darmmicrobiota die aan een enkele bron van voedingsvezels aangepast zijn, zich mogelijk minder goed aan andere vezels kunnen aanpassen. Ten slotte wordt de mogelijke invloed van deze studie op onderzoek, productie en consumptie van voedingsvezels beschreven.

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About the Author

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Curriculum vitae



Melliana Christina Jonathan was born in Semarang, Central Java, Indonesia on 4 May 1979. She studied Biology, with a focus on Industrial Microbiology at Satya Wacana Christian University, Salatiga, Central Java, Indonesia. Her bachelor study was finished in 2002. Afterwards, she worked in PT. Pulau Sambu Guntung, a coconut product manufacturing company located in the province of Riau, Indonesia. She enjoyed working in the Research and Development department of the company and became interested in the field of food technology. Four years later, in 2006, she was awarded with a scholarship to study Food Technology with a focus on Product Functionality in Wageningen University, Wageningen, The Netherlands. She did her master thesis on the characterisation of two acetylxylylan esterases and an α -glucuronidase at the Laboratory of Food Chemistry. Her minor thesis was performed at FrieslandFoods (now FrieslandCampina) R&D Centre in Deventer. After finishing her master study in 2008, she obtained an opportunity to conduct a PhD research in the Laboratory of Food Chemistry, Wageningen University. The results of the research are described in this thesis. Melliana is now working temporarily in the same department as a researcher.

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List of publications

Jonathan, M. C.; Bosch, G.; Schols, H. A.; Gruppen, H., Separation and identification of individual alginate oligosaccharides in the feces of alginate-fed pigs. *Journal of Agricultural and Food Chemistry*. DOI: 10.1021/jf304338z.

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Pouvreau, L.; **Jonathan, M. C.**; Kabel, M. A.; Hinz, S. W. A.; Gruppen, H.; Schols, H. A., Characterization and mode of action of two acetyl xylan esterases from *Chrysosporium lucknowense* C1 active towards acetylated xylans. *Enzyme and Microbial Technology* **2011**, 49, 312-320.

Overview of completed training activities

Discipline specific activities

Courses

Summer course glycosciences (VLAG), incl. poster, Wageningen, The Netherlands, 2010

Advanced food analysis (VLAG), incl. poster, Wageningen, The Netherlands, 2010

Food fermentation (VLAG), incl. poster, Wageningen, The Netherlands, 2012

Conferences

Dietary fibre conference and workshop fibre analysis, Vienna, Austria, 2009

Cell wall meeting, incl. oral presentation, Porto, Portugal, 2010

EPNOE meeting, incl. oral presentation, Wageningen, The Netherlands, 2011

Plant and seaweed polysaccharide symposium, incl. oral presentation, Nantes, France, 2012

General courses

PhD introduction week (VLAG), Bilthoven, The Netherlands, 2009

Techniques for writing and presenting a scientific paper, Wageningen, The Netherlands, 2009

Teaching and supervising thesis students, Wageningen, The Netherlands, 2009

Effective behaviour in your professional surroundings, Wageningen, the Netherlands, 2011

Project and time management, Wageningen, The Netherlands, 2012

Career perspectives, Wageningen, The Netherlands, 2012

Optionals

- PhD research proposal
- Food Chemistry study trip, Ghent Belgium 2009
- Food Chemistry PhD trip, Switzerland and Italy 2010
- Food Chemistry PhD trip, Singapore and Malaysia 2012
- Food Chemistry Seminars and Colloquia 2008-2012

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Melliana Jonathan, 2013

