Fate of rapeseed meal polysaccharides during digestion in pigs and poultry

Effect of processing and enzyme addition

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Thesis

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Abstract

In this thesis, the fate of non-starch polysaccharides (NSP) from rapeseed meal (RSM) during fermentation *in vitro* and *in vivo* was studied. The aim was to understand and improve the fermentation of NSP from RSM in poultry and pigs, by processing and enzyme addition. First, the NSP-structures in RSM were characterized as being branched arabinan, arabinogalactan type II, homogalacturonan, glucurono-xylan, XXGG- and XXXG-type xyloglucan, and cellulose. Second, RSM was processed using shear, heat, and acid prior to *in vitro* incubation, in the presence or absence of pectolytic enzymes. Acid-treatment combined with pectolytic enzymes was the best option to improve NSP-solubilization *in vitro*. Unprocessed and acid-extruded RSM with or without addition of enzymes were fed to broilers. In broilers, 22% of the NSP in unprocessed RSM could be fermented, which only significantly improved to 38% by addition of commercial pectolytic enzymes. In broilers' excreta, XXXG-type xyloglucan, (glucurono-)xylan, arabinan, and cellulose remained unfermented. Unprocessed and acid-extruded RSM was also fed to growing pigs and NSP-fermentation was followed along the digestive tract. In pigs, at the terminal ileum 22% of the NSP was cumulatively fermented and total tract around 70% was fermented. Acid-extrusion improved total tract NSP-fermentability in pigs numerically by 4% points. Water-soluble carbohydrates were nearly completely fermented. In the feces some rhamnogalacturonan, (branched) arabinan, linear xylan, XXXG-type xyloglucan, galactomannan, and cellulose remained. Surprisingly, during alkaline extraction of the broilers' excreta and pigs' feces, around 40% (w/w) of the insoluble carbohydrates was released as glucosyl- and/or uronyl-rich carbohydrates, probably originally present via ester-linkages or hydrogen-bonding within the cellulose-lignin network. These linkages are expected to hinder complete NSP-fermentation.

List of abbreviations

Ara	Arabinose
ASS	Alkali soluble solids
Br	Branched glycoside
ChSS	Chelating agent soluble solids
DASS	Dilute alkali soluble solids (50mM NaOH)
DM	Dry matter
DMCV	Dry matter cumulative volume
DP	Degree of polymerization
FCR	Feed conversion ratio
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GIT	Gastro-intestinal tract
GC	Gas chromatography
Glc	Glucose
GlcA	Glucuronic acid
GMD	Geometric mean diameter
Hex	Hexose
HG	Homogalacturonan
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
HPSEC	High performance size exclusion chromatography
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
Man	Mannose
MS	Mass spectrometry
na	Not analyzed
NSP	Non-starch polysaccharides
PAD	Pulsed amperiometric detection
RES	Residue after sequential extraction
RG	Rhamnogalacturonan
Rha	Rhamnose
RSM	Rapeseed meal
T	Terminal linked glycoside
Tr	Traces
TTAD	Total tract apparent digestibility
UA	Uronic acid
WBC	Water binding capacity
WSS	Water soluble solids
WUS	Water unextractable solids
Xyl	Xylose

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Chapter 1

General introduction

1.1 Project outline

The animal feed industry has been confronted with an increase in costs of raw materials. This is due to increased demands of these raw materials, next to their use as animal feed ingredients. Examples are grains and oil seeds, of which starch and oil, respectively, are used for both biofuel production and human consumption. The co-products of these materials are relatively cheap and abundant. Therefore, they are used in animal feed mostly for their high protein content. However, these co-products are also high in non-starch polysaccharides (NSP), which cannot be degraded by the animal's own digestive enzymes.

This PhD-thesis is part of a project entitled "Improved utilization of complex carbohydrates in pig and poultry by novel technologies". The project aim is to improve the NSP-utilization of co-products from grain ethanol and rapeseed oil in animal feed in order to reduce feed costs and enhance the sustainable use of feed resources. Hereto, the structure of the complex carbohydrates present in these co-products needs to be understood and insight needs to be gained in the utilization of these complex carbohydrates in animals. Both processing technologies and enzyme addition are considered in this project to improve the digestibility and fermentability of the co-products.

As part of the larger project, this thesis focuses on the characterization of NSP-structures in rapeseed meal, being an important oil seed, and their fate during fermentation in poultry and pigs. It is expected that an indication of which NSP-structures are recalcitrant in poultry and pigs is obtained, which may aid to improve the NSP utilization. This is tested both in an *in vitro* assay and in two *in vivo* trials. In addition, the effect of processing and enzyme technologies on the NSP-structures and their fermentation was examined.

1.2 Rapeseed oil co-products

Seed oil co-products that are commonly used in animal feed remain from the oil production from e.g. soybean, rapeseed, and sunflower. These co-products are mostly used as protein-source in feed in the form of meals. The oil is used for both human consumption and biofuel (*1*).

In Europe, in 2008/2009, 17% of the oil seed meals used in feed is derived from rapeseed, next to 69% from soybean, and 9% from sunflower (*2*). Rapeseed meal (RSM) is cheap and

increasingly abundant. Therefore, in this thesis the digestion of RSM is evaluated during various digestion trials. Rapeseed is a yellow-flowering plant belonging to the *Brasicaceae* family. The rapeseed used in Europe nowadays is a *Brassica napus* species, which is a cross-breed between *Brassica campestris* and *Brassica oleracea* (*3*). The seeds resulting from this cross-breed are known to be low in erucic acid and glucosinolates, which are anti-nutritive factors for humans and animals (*4*). This rapeseed being low in anti-nutritive factors is also referred to as "double low" or "00".

For oil production, seeds are cleaned, preconditioned at 30-40°C for 30-45 min, and flaked to (partly) rupture the cell walls. The flakes are heated at 65-85°C for 20-40 min to inactivate enzymes present in the seeds. The cooked flakes are screw-pressed to extract 60-70% of the oil. After solvent extraction (50-60°C) of the rest of the oil, the meal is dried by toasting (105°C, 30-40 min) (*5*).

RSM is composed of around 38% (w/w) protein (*6*) and 16-22% non-starch polysaccharides (*7*). Other components are expected to be lignin, ash, and lipids (*6*). No starch is present in RSM.

So far, cell wall polysaccharides from *Brassica napus* have not been studied in detail. But, polysaccharides reported in meal from *Brassica campestris* are homogalacturonan, branched arabinan, arabinogalactan, xyloglucan, xylan, and cellulose (*8, 9*). So, RSM is considered to be a pectin-rich raw material. In general, such cell wall polysaccharides are not (completely) fermented by monogastric animals.

1.3 Plant cell walls

Plant tissues, like rapeseed, are composed of plant cells. These plant cells obtain their firmness from surrounding cell walls. Other functions of plant cell walls are protection, involvement in cell size and shape, and control of rate and direction of cell growth (*10*).

1.3.1 Cell wall architecture

Plant cell walls are composed of a primary and a secondary layer, which are both built from various polymers, like polysaccharides, lignin, and protein. Polysaccharides contribute the most to the cell wall composition (Figure 1.1). The primary layer is a flexible layer formed when the cell is still capable of growing (*11*). Primary cell walls are predominantly composed of polysaccharides (up to 90% of the dry matter), with smaller

amounts of structural glycoproteins (2-10%), phenolic esters (<2%), ionically and covalently bound minerals (1-5%), and enzymes (*12*).

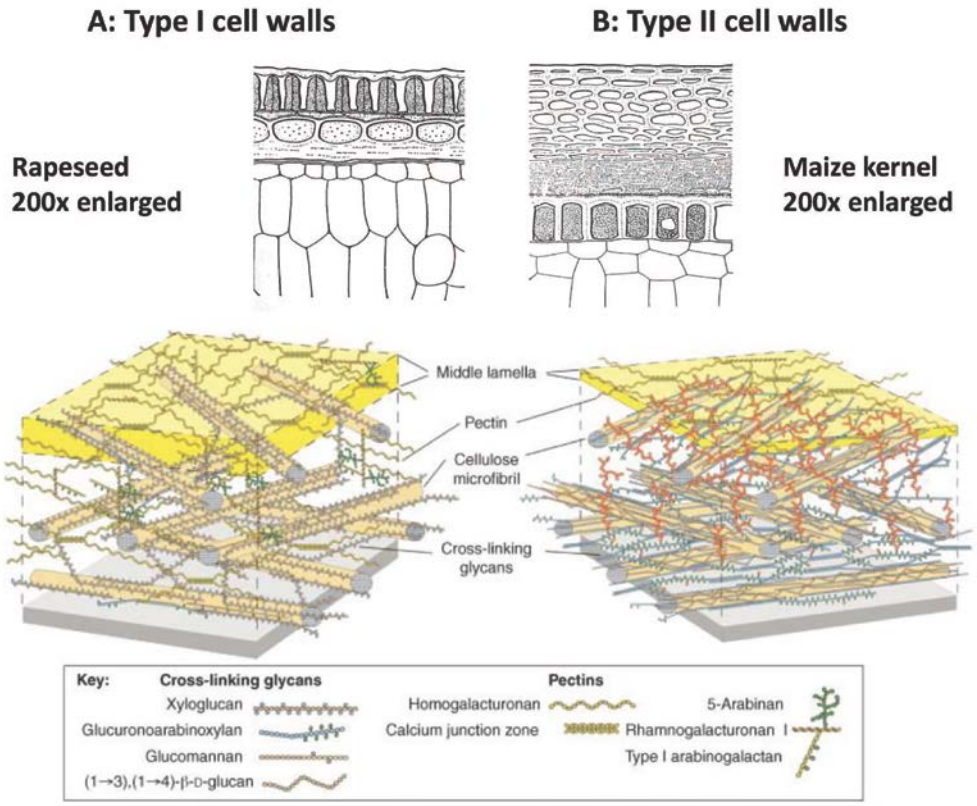

Figure 1.1. Plant cell wall in type I primary cell walls (e.g. rapeseed, A) and type II cell walls (e.g. maize, B). Microscopic pictures from Gassner et al. (*13*). Cell wall figures from Carpita & McCann (*14*).

In general, there are two types of primary cell walls (Figure 1.1). Type I primary cell walls, which contain typically pectin and xyloglucan, are found in dicotyledons (e.g. oil seeds), non-graminaceous monocotyledons, and gymnosperms (*15*). Type II primary cell walls, are present in the Poaceae or Gramineae (grains), are rich in arabinoxylan, and contain <10% pectin (*16*). These plants typically have a thicker secondary cell walls than plants with Type I primary cell walls (*14*). In the primary cell wall, cellulose microfibrils are interlinked with xyloglucan (Type I) or xylan (Type II) via hydrogen bonds forming a stiff network (*11, 15*).

Pectins or xylans together with structural proteins are physically entangled within this network (*10*). Pectins have been shown to be covalently linked to each other (*17*) and cross-links between pectin and hemicellulose (*18*), and between pectin and cellulose (*19*) have been suggested.

After cell growth has ceased, the secondary cell wall has been formed by the deposition of other polymers, such as lignin and subarin, next to mainly polysaccharides (*20*). In secondary cell walls of monocotyledonous plants, the main carbohydrates are glucuronoarabinoxylan and cellulose, while in dicotyledonous plants, the main carbohydrates are 4-O-methylglucuronoxylan, xyloglucan, and cellulose (*21*).

1.3.2 Plant cell wall polysaccharides

1.3.2.1 Pectins

Pectins are a diverse group of polysaccharides. Structures that belong to this group of polysaccharides are: homogalacturonan (HG), rhamnogalacturonan I and II (RG-I and RG-II), xylogalacturonan (XGA), arabinan, and arabinogalactan type I and II (AG-I and AG-II). Structures are schematically represented in Figure 1.1. The composition, abundance, and properties of these pectins vary with source and developmental stage.

HG is a linear polymer of galacturonic acid units linked through α-1,4-glycosidic linkages. HG can be substituted with methanol esters at C6 and acetic acid esters at the O2 and/or O3-positions (*22*).

XGA has an HG backbone, substituted with single units of β-1,3-linked xylosyl units. It can carry methanol ester groups at the galacturonic acid residues (*23*).

RG-I consists of a backbone of α-1,4-linked galacturonic acid residues alternating with α-1,2-linked rhamnosyl residues. The O4-position of the rhamnosyl units can be linked to an arabinan or arabinogalactan side chain. The galacturonic acid can be esterified with acetic acid at O2 and/or O3 (*24*).

RG-II consists of 7-9 α-1,4-linked galacturonic acid residues substituted with complex side chains containing 12 different monosaccharides, including some rare ones. RG-II is able to form dimers via a borate ester, which results in cross-linking of pectin molecules within the cell wall (*25*).

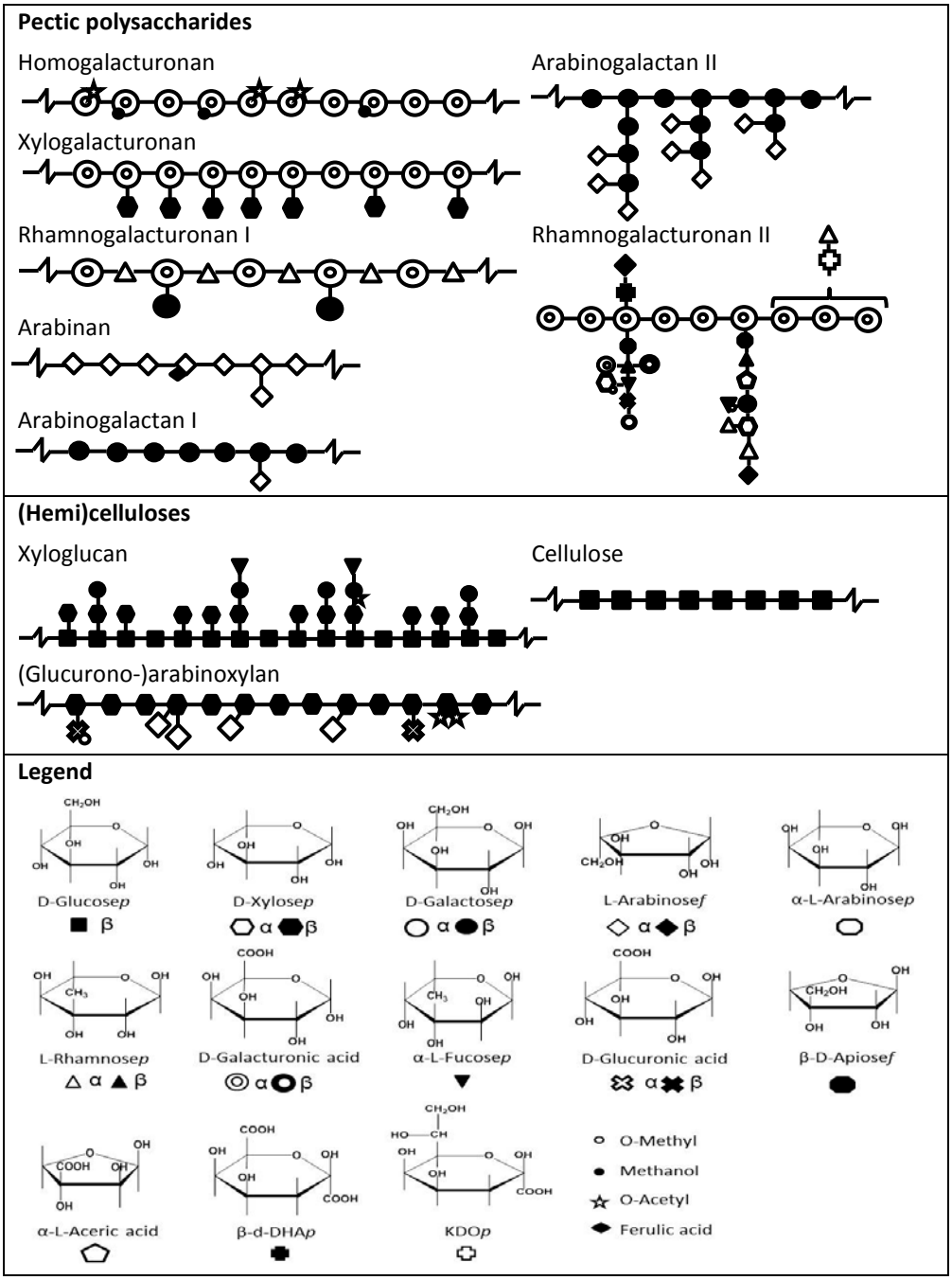

Figure 1.2. Schematic representation of NSP-structures. DHA: 3-deoxy-D-manno-2-octulosonic acid; KDO: 3-deoxy-D-lyxo-2-heptulosaric acid.

Arabinan consists of a backbone of α-1,5-linked arabinosyl residues, which are usually substituted with arabinosyl units by α-1,2 or α-1,3-linkages. The arabinosyl residues can be feruloylated at O2 and/or O5 (*26*).
AG-I is composed of a β-1,4-galactosyl backbone, which is substituted with α-1,5-linked arabinosyl units at the O3-position (*15*).
AG-II is composed of a β-1,3-galactosyl backbone, which is substituted with β-1,6-galactosyl units. Substituents with single arabinosyl residues can occur. AG-II is shown to be linked to proteins forming arabinogalactan proteins (*27*).

1.3.2.2 Hemicelluloses

Hemicellulose structures that are present in plant cell walls are xylans, xyloglucans, β-glucans, and mannans (Figure 1.1).
Xylans are the main hemicellulose in monocotyledonous plants (*16*). They consist of a β-1,4-xylosyl backbone, which can be substituted with arabinosyl residues (arabinoxylan), 4-O-methyl-glucuronic acid (glucuronoxylan), or both (glucurono-arabinoxylan). Xylans can be esterified with acetic acid at the O2- and/or O3-position and can also be feruloylated at the arabinosyl residues (*28*).
Xyloglucans are the main hemicellulose in dicotyledonous plants (*29*). Xyloglucans are composed of a β-1,4-glucosyl backbone, which can be substituted with xylosyl residues at the O6- position. The xylosyl residues can be further substituted with galactosyl, fucosyl, and arabinosyl units. Xyloglucans can be classified as XXGG-, XXXG-, or XXXGG-type (*30*). Three domains of xyloglucan are known: (a) xyloglucan present in free loops and cross-links, which are enzyme accessible, (b) xyloglucan linked via hydrogen bridges to the surface of cellulose microfibrils, which is extractable with concentrated alkali, but not enzyme accessible, and (c) xyloglucan entrapped in the amorphous cellulose fibrils, which is not extractable by concentrated alkali and only accessible for enzymes when cellulose is co-degraded (*31*).
β-*1,3-1,4-Glucans* are a linear polymer composed of a glucosyl backbone. Due to the mixed linkages, β-1,3 and β-1,4, the conformation is more extended and less ordered than cellulose, which increases solubility.
Mannans have a backbone of β-1,4-linked mannosyl residues, which may be interrupted by glucosyl units and may be substituted with a single α-1,6-linked galactosyl unit (*32*).

1.3.2.3 Cellulose

Cellulose is the principle cell wall polysaccharide of higher plants and, therefore, the most abundant carbohydrate. Cellulose is a linear, water-insoluble homopolymer of repeating β-1,4-glucosyl units (Figure 1.1). Individual cellulose chains lie side by side in bundles, held together by hydrogen bonding between the numerous neighboring OH-groups to form a ribbon-like two-fold helix. Multiple chains can aggregate by intermolecular hydrogen-bonds to form microfibrils (*33*).

1.4 Non-starch polysaccharides in animal nutrition

The animals own enzymes are able to digest starch, fat, and protein, but they are not able to degrade the non-starch polysaccharides (NSP; Section 1.3). Nevertheless, the NSP can be fermented by microbiota in the gastro-intestinal tract. Inclusion of NSP in the diet also influences digesta transit, bulking properties (*34*), microbial activity (*35-37*), and gut physiology and function (*37-40*). Altered physical properties of digesta can influence digestion of other nutrients. Also, NSP is shown to encapsulate other nutrients, thereby limiting accessibility of these nutrients for digestion (*35*).

1.4.1 Current analysis in animal nutrition research

In animal nutrition research, NSP are usually analyzed gravimetrically as crude fiber, acid detergent fiber, and neutral detergent fiber (*41*) or calculated as dry matter minus the sum of crude protein, crude fat, ash, starch, and ethanol-soluble sugars (*42*). Figure 1.3 shows a schematic representation of the division of dietary carbohydrates and fibers. In carbohydrate chemistry research, other analysis techniques for the accurate determination of various groups of complex carbohydrates are available (*43-46*). This thesis will combine knowledge on such detailed carbohydrate analysis with digestion studies.

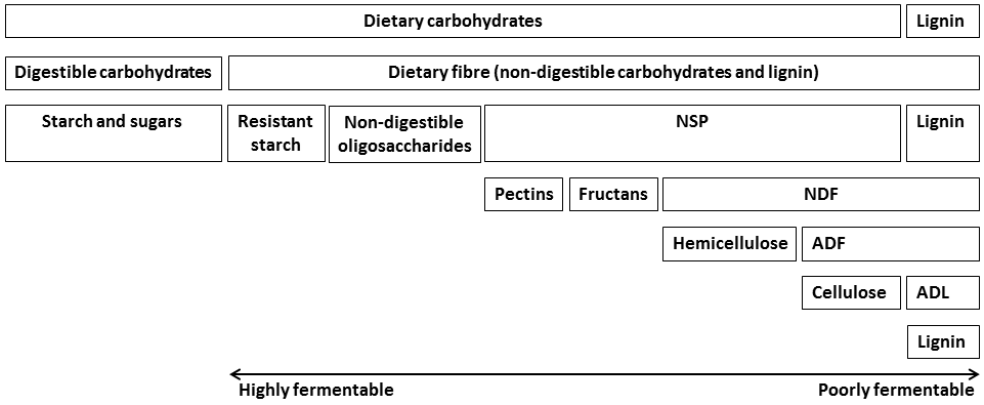

Figure 1.3. Schematic representation of dietary carbohydrate, fiber, and NSP-composition (*47*). NSP: Non-starch polysaccharides, ADF: Acid Detergent Fiber, NDF: Neutral Detergent Fiber, ADL: Acid Detergent Lignin.

1.4.2 Poultry

A schematic digestive tract of chicken is shown in Figure 1.4. Poultry cannot chew their feed. Therefore, they developed a special organ, the crop, where the food is stored and moistened. Then, the proventriculus serves as a glandular stomach, where gastric juices and enzymes start the degradation of feed. The gizzard serves as a muscular stomach where the food is mechanically further broken down. The small intestine follows, where enzymatic digestion of small carbohydrates, proteins and lipids, and absorption of their degradation products takes place.

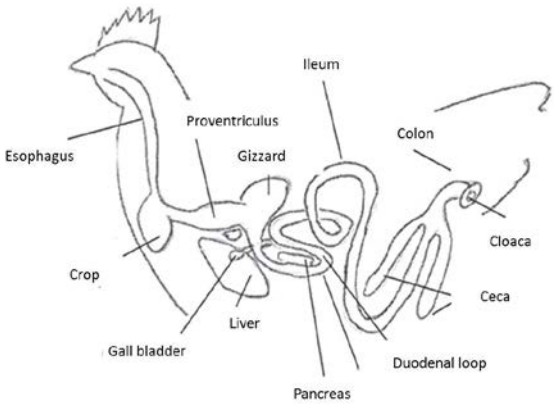

Figure 1.4. Schematic digestive tract of poultry.

The extent of digestion is largely driven by the transit time in the small intestine (*48*). After the small intestine, the ileocecal valve opens regularly to let digesta enter the colon. Gentle refluxive peristalsis washes digesta with urine from cloaca, while pushing the soluble fraction and small particles into the ceca (*49*). Some soluble non-digestible carbohydrates are fermented by the microbiota in the ceca (*50*), resulting in the production of short chain fatty acids (SCFA), lactate, and gases (*51*). The large intestine is very short, but some microbial fermentation takes place to finalize nutrient recovery, prior to defecation (*48*).

1.4.3 Pigs

A schematic digestive tract of pigs is shown in Figure 1.5. Feed directly enters the stomach, where gastric juices are produced to start degradation. Further degradation and digestion takes place in the small intestine, which is divided into duodenum, jejunum, and ileum. The digesta are mixed with secretions from the duodenum, liver, and pancreas. Pancreatic enzymes can degrade part of the carbohydrates, mostly starch (*52*), fat, and proteins, which can be absorbed in the jejunum. At the end of the ileum some fermentation can occur. The large intestine serves to absorb nutrients, electrolytes and water from the digesta. In the cecum and start of the colon, NSP can be (partly) fermented by gut microbiota, yielding short chain fatty acids (SCFA), lactate, microbial biomass, and gases (*53*). SCFA are absorbed in the intestine and thereby serve as an energy source for the animal.

NSP fermentation varies depending on the individual NSP-structures. In general, pectins and hemicelluloses are further fermented than cellulose (*54*).

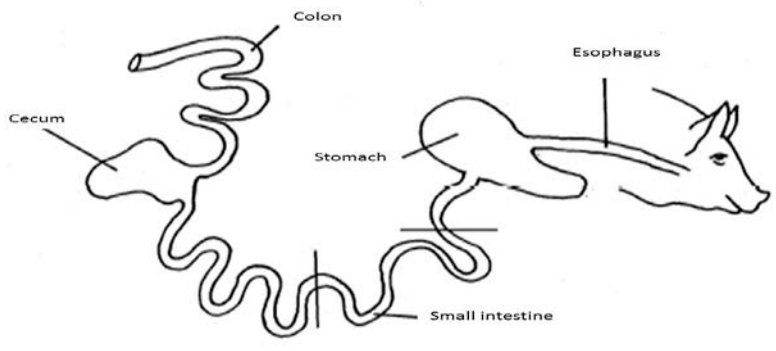

Figure 1.5. Schematic digestive tract of pig.

1.4.4 RSM in diets for poultry and pigs

For poultry, the fermentability rate of NSP from RSM is 14% (*55*), which is slightly lower compared with other protein sources used in poultry feed, like soybean meal, from which 20% of the NSP can be fermented by poultry (*55*). The limiting step in addition of RSM to poultry diets is the high NSP-level, since (soluble) NSP increase digesta viscosity and thereby limit digestion of other nutrients (*56*).

For pigs, the fermentability rate of NSP from RSM is 58% (*55*), which, like for poultry, is relatively low compared with other protein-sources in pig feed, as sugar beet pulp and soybean meal, from which 85% and 84% of the NSP can be fermented by pigs, respectively (*55*). The low fermentability rate of NSP from RSM indicates that there is still more energy available in RSM, which could potentially be used by the animal besides the protein.

1.5 Feed processing

The utilization of feed by animals is known to improve by processing, such as milling or heat treatment. In general, information on the influence of treatments on the NSP- and/or fiber-fermentability is available, but these studies do not describe the fermentation of specific carbohydrate structures in detail. Treatments used in animal feed industry are listed in Table 1.1 (poultry) and Table 1.2 (pigs) for their effect on NSP- and/or fiber-fermentability, also recently reviewed (*57*). In these tables NSP-fermentability us based on analysis currently used in animal research as described in Section 1.4.1 of this thesis.

1.5.1 Enzymatic treatment

Addition of NSP-degrading enzymes to feed for poultry and pigs has increased at a commercial scale during the last 25 years (*56*). Addition of NSP-degrading enzymes can help to open up the cell wall material and make it more accessible for fermentative enzymes, especially important for pigs (*58*). In addition, enzymes can reduce digesta viscosity and its detrimental effects on nutrient digestion and absorption by degrading viscous polysaccharides, which is especially important in poultry (*56*), also counteracting the increase in viscosity due to solubilization of NSP during technological pretreatment. Finally, enzymes can produce specific low molecular weight carbohydrates, which have prebiotic properties in animals (*56*). In this thesis the first two options are aimed at by the addition of enzymes. Research has shown that a combination of different enzyme

activities is required to improve NSP-utilization both in poultry and in pigs (*59*). Addition of multicarbohydrase enzyme mixtures, containing cellulase, xylanase, glucanase, and/or pectinase activity, to RSM-containing diets improved NSP-fermentability in poultry (*7, 60, 61*). Also in pigs, addition of multicarbohydrase enzyme mixtures to RSM-containing diets improved NSP-fermentability (*59, 62*).

A difficulty in summarizing results of enzyme addition on the NSP-fermentation in pig and poultry diets, is that in quite some studies the enzyme activities used are not specific for the degradation of the specific carbohydrate structures present in the feed. Therefore, enzyme addition is not part of the Tables 1.1 and 1.2.

1.5.2 Mechanical treatment

Mechanical treatments are for example milling and flaking, of which milling is most commonly used in feed industry. During milling, the surface of the particles increases, thereby increasing the extractability of polysaccharides and the accessibility for digestive and microbial enzymes. Reduction in particle size has shown to improve growth performance of pigs and to improve fermentability of the fiber fraction in poultry and pigs (Tables 1.1 and 1.2). The size distribution also influences the physical properties of the digesta, like water binding capacity and viscosity, and thereby the fate of components in the gut. As an example, hammer-milling of peas improved NSP-fermentability in broilers (*63*). In growing pigs, hammer-milling improved NSP-fermentability from wheat, but hammer-milling of barley did not have an effect on NSP-fermentability (*64*).

1.5.2 Thermal treatment

In the animal feed industry, a wide variety of thermal treatments are commonly used, e.g. extrusion, expansion, toasting, and pelleting. While mechanical treatments are usually performed on a single ingredient, thermal treatments are generally performed on the whole feed. Thermal treatment of single ingredients may have great potential over treatment of the whole feed, since the presence of other ingredients may decrease effectiveness of extrusion. The presence of lipids, for example, will result in a lower development of shear forces (*65*).

Table 1.1. Treatments used in animal feed industry, based on either mechanical and/or thermal effects and their effect on total tract NSP-fermentability in poultry.

Treatment	Mechanical	Thermal	Age [a]	Effect on NSP-fermentability [b]	Reference
Expansion	x	x	28	Barley: +	(*66*)
Extrusion	x	x	25	Soybean meal: ++	(*67*)
Hammer-milling	x		17	Peas: ++	(*63*)
Infrared irradiation		x	25	Barley: 0	(*66*)
Steam cooking-flaking		x	4-21	Barley: ++ [c]	(*68*)

[a] Age of animal in days. [b] Change in total tract fermentability of the processed diet compared with untreated control diet expressed as – (6-10% units decrease), - (2-6% units decrease), 0 (-2 till +2% units), + (2-6% units increase) and ++ (6% units or more increase). [c] Based on ADF/NDF-analysis.

Table 1.2. Treatments used in animal feed industry, based on either mechanical and/or thermal effects and their effect on fecal NSP- or fiber-fermentability in pigs.

Treatment	Mechanical	Thermal	Age class [a]	Effect on NSP-fermentability [b]	Reference
Baking		x	Finishers	Barley: ++	(*69*)
Expansion	x	x	Growers	Barley: +	(*70*)
			Growers	Peas: ++	(*71*)
			Growers	Barley/wheat products: 0 [c]	(*72*)
Extrusion	x	x	Piglets	Barley: + [c]	(*73*)
			Growers	Barley: --	(*74*)
			Finishers	Barley: +	(*75*)
			Growers	Peas: +	(*74*)
			Growers	Wheat bran/potato starch: +++	(*74*)
			Piglets	Maize: 0 [c]	(*73*)
Hammer-milling	x		Growers	Barley: 0 [c]	(*64*)
			Growers	Wheat: + [c]	(*64*)
Infrared irradiation		x	Piglets	Maize: 0 [d]	(*76*)
Pelleting	x	x	Growers	Barley: +	(*70*)
			Finishers	Barley: -	(*58*)
			Growers	Peas: + [c]	(*77*)
Steam cooking		x	Piglets	Barley: + [d]	(*78*)
			Piglets	Maize: 0 [d]	(*76*)
Toasting		x	Piglets	Barley: -- [c]	(*79*)
			Growers	Peas: -	(*80*)

[a] Age class of animal defined as piglets (<20 kg), growers (20-50 kg), and finishers (>50 kg). [b] Change in total tract fermentability of the processed diet compared with untreated control diet expressed as – (6-10% units decrease), - (2-6% units decrease), 0 (-2 till +2% units), + (2-6% units increase) and ++ (6% units or more increase). [c] Based on ADF/NDF-analysis.

During extrusion the product is exposed to elevated temperature (90-160˚C) for a short time (30-120s) at high pressures, and at relatively low moisture contents (below 30%) (81). Extrusion of soybean meal improved fecal NSP-fermentability considerably in poultry (Table 1.1), while extrusion of barley, peas, and wheat bran slightly improved fecal fermentability in pigs (Table 1.2). On the other hand, extrusion of barley showed to decrease NSP-fermentability in growing pigs. Expansion is a process similar to extrusion, but the mechanical energy is much higher and, therefore, a lower temperature is sufficient to obtain similar effects (82). Expansion of barley slightly improved NSP-fermentability in poultry and pigs, and considerably improved NSP-fermentability of peas in growing pigs. Expansion of a diet containing barley and wheat products did not have an effect on NSP-fermentability.

Toasting is mostly performed after oil extraction. In this step the extraction solvent is removed. During this process anti-nutritive factors are inactivated, protein digestibility can be influenced and the Maillard reactions can take place (*81*). Toasting of barley and peas seems to decrease NSP-fermentability in piglets and growing pigs.

After pre-treatment of the feed it can be pelleted. Pelleting is a combination of moisture, heat, and mechanical pressure to agglomerate diets. It results in a significant increase in crude fiber-fermentability in growing pigs fed with a diet containing barley, wheat, and soybean meal (*82*). For finisher pigs, pelleting of barley does not seem to be beneficial (*58*).

1.5.3 Chemical treatment

In poultry and pig nutrition, chemical treatments have not been used at an industrial scale, and are, therefore, not included in Tables 1.1. and 1.2. Nevertheless, mild acid treatment of rye, combined with a thermal treatment in an autoclave, showed to alleviate growth-depressing properties of rye in chicks and especially improve the release of arabinosyl residues (*83*). In addition, pre-treatment with sulfuric acid has been shown to increase extractability of carbohydrates from lignocellulosic biomass (*84*). Recently, also dicarboxylic organic acids at elevated temperatures have been shown to increase carbohydrate solubility from plant material, e.g. corn stover (*85*). Dicarboxylic acids have preference over sulfuric acid in animal nutrition, since no additional sulfates are introduced into the diet.

In diets for ruminants, organic acids, like malic acid, are added because of their positive effect on ruminal fermentation and the ability to substitute antimicrobial compounds, such as monensin (*86*).

1.6 Terminology used in this thesis

In this thesis the fate of cell wall polysaccharides from RSM is studied. Due to the action of enzymes, degradation of cell wall polysaccharides takes place. Depending on the experimental set-up the terms solubilization, digestion, and fermentation are used.

In Chapter 2, the cell wall polysaccharides are characterized. These are referred to as NSP in the successive chapters.

During the *in vitro* study (Chapter 3), simulating the pig's upper gastro-intestinal tract, actually solubilization of NSP was analyzed. *In vivo* starch, protein, and fat can be degraded by the animals' digestive enzymes and are, therefore, referred to as being digested. Animals lack the digestive enzymes to degrade NSP, but they are degraded by enzyme produced by the microbiota in the intestine and are, therefore, referred to as being fermented. The remained are called unfermented carbohydrates.

1.7 Aim and thesis outline

The rate-limiting step in NSP-utilization from RSM by poultry and pigs was not known, since the unfermented carbohydrate structures have not been studied in detail so far. Therefore, the aim of this thesis was to characterize rapeseed meal polysaccharides and study their fate during fermentation in poultry and pigs. Also, technological treatments and/or enzyme addition to improve utilization of non-starch polysaccharide fermentability were explored.

Chapter 2 describes the characterization of non-starch polysaccharides (NSP) in rapeseed meal. Cell wall polysaccharides were extracted using different solvents and analyzed for their carbohydrate compositions and polymeric structures by enzymatic fingerprinting and linkage type analysis. In **Chapter 3** the *in vitro* solubilization of rapeseed meal polysaccharides, which is processed using different technological and enzymatic treatments, is described. The aim of this part was to select the best (combination of) technological treatment and enzyme addition for the further *in vivo* trials. The composition of residual carbohydrates after *in vitro* solubilization was determined and

related to the NSP originally present in RSM. In addition, physical characteristics as water binding capacity and viscosity were analyzed.

Chapter 4 describes the *in vivo* digestion of rapeseed meal in poultry. The effect of the selected technological treatments and enzyme addition on NSP-fermentability was studied in broilers. In **Chapter 5**, a selection of fecal samples from broilers fed with acid-extruded rapeseed meal (with and without the addition of enzymes) was further analyzed. Unfermented carbohydrates were extracted from the feces and analyzed for their carbohydrate composition and polymeric structure and related to the NSP originally present in RSM.

In **Chapter 6** remaining carbohydrates after *in vivo* digestion of (processed) rapeseed meal in pigs were explored. Samples from different parts of the digestive tract were analyzed for carbohydrate composition and polymeric structures. In **Chapter 7** the results of the different phases of this project are summarized and discussed. The predictability of *in vivo* fermentation of RSM NSP by the *in vitro* assay was evaluated and unfermented carbohydrate structures in broilers' and pigs' feces are compared.

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Chapter 2

Characterization of cell wall polysaccharides from rapeseed (*Brassica napus*) meal

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Abstract

To enable structural characteristics of individual cell wall polysaccharides from rapeseed (*Brassica napus*) meal (RSM) to be studied, polysaccharide fractions were sequentially extracted. Fractions were analyzed for their carbohydrate (linkage) composition and polysaccharide structures were also studied by enzymatic fingerprinting.

The RSM fractions analyzed contained pectic polysaccharides: homogalacturonan in which 60% of the galacturonic acid residues are methyl-esterified, arabinan branched at the O-2 position and arabinogalactan mainly type II. This differs from characteristics previously reported for *Brassica campestris* meal, another rapeseed cultivar. Also, in the alkali extracts hemicelluloses were analyzed as xyloglucan both of the XXGG- and XXXG-type decorated with galactosyl, fucosyl and arabinosyl residues, and as xylan with O-methyl-uronic acid attached. The final residue after extraction still contained xyloglucan and remaining (pectic) polysaccharides next to cellulose, showing that the cell wall matrix of RSM is very strongly interconnected.

2.1 Introduction

Rapeseed (*Brassica napus*) meal (RSM) is a by-product from the production of rapeseed oil and is used as animal feed. The high demand for energy has lead to increased production of bio-diesel and as a consequence more RSM has become available for the animal feed industry. Nowadays in Europe, 24% of oilseed meal used in feed originates from rapeseed compared with 59% originates from soybean and 12% from sunflower (*1*). A drawback of RSM is that, compared to soybean meal, it is high in cell wall polysaccharides, which cannot be degraded by endogenous enzymes of monogastric animals and improvement of digestibility by pre-treatment of RSM is needed (*2*). However, knowledge on cell wall polysaccharide structure in RSM is currently limiting.

Oil seeds are dicotyledonous plants. They are rich in polysaccharides, like pectins, hemicelluloses and cellulose, which together form a complex network within the plant cell wall. Pectins are a very diverse group of polysaccharides consisting of the structural elements homogalacturonan (HG), rhamnogalacturonan I (RG-I) and II (RG-II), and xylogalacturonan. HG is a linear polymer of α-1,4-linked galacturonic acid units, which can be methyl-esterified on the O-6 position. Also, they can carry acetyl-groups on the O-2 and/or O-3 position (*3*). RG-I consists of a backbone of alternating rhamnosyl and galacturonyl residues, to which neutral side chains, such as arabinans and arabinogalactans, are proposed to be attached (*3*). The hemicelluloses in oil seeds are mainly xyloglucans, which are polysaccharides with a β-1,4-linked glucosyl backbone with 1,6-linked xylosyl units, to which galactosyl and fucosyl residues can be attached.

Rapeseed derives from several species belonging to the genus Brasicaceae. In this paper *Brassica napus* is studied, of which the cell wall polysaccharides have not been characterized so far. *B. napus* is a crossbreed between *B. campestris* and *B. oleracea*. From all three cultivars rapeseed is grown and used in industry (*4*). Characterization of polysaccharides from *B. campestris* meal has been published (*5-10*). From the *B. oleracea* cultivar, only the cell wall polysaccharides from its cabbages have been analyzed (*11*), and not from the meal of its seeds.

The aim of this study was to characterize cell wall polysaccharides from *B. napus* meal, which will help to understand and improve the limited digestibility of these polysaccharides in this abundant by-product for monogastric animals.

2.2 Materials and methods

2.2.1 Plant material

Rapeseed meal (ADM, Hamburg, Germany, 2009) was provided by Nutreco Nederland B.V. (Boxmeer, The Netherlands).

2.2.2 Isolation of water unextractable solids (WUS) and sequential extraction

Rapeseed meal (RSM) was ground using a Retsch mill (Retsch, Haan, Germany) to pass a 0.5 mm sieve. This meal (40 g) was extracted three times with 1 L of demineralized water during 1 hour at 70˚C. After each extraction, solubles were separated from the insoluble residue by filtration on a G2-glass filter. Water soluble solid (WSS) fractions were combined and (without previous dialysis) freeze-dried.

Water Unextractable Solids (WUS) were sequentially extracted (25 mL extractant per gram material) starting with 0.05 M 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) and 0.05 M ammonium-oxalate in 0.05 M NaOAc (pH 5.2) at 70°C (Chelating Agent Soluble Solids, ChSS). Chelating agent unextractable solids were further extracted with 0.05 M NaOH containing 20 mM $NaBH_4$ at 4°C (Dilute Alkali Soluble Solids, DASS). Again, the unextracted solids were further extracted with 4 M NaOH containing 20 mM $NaBH_4$ at 4°C (4 M Alkali Soluble Solids, 4MASS). Similarly, extraction with 6 M KOH containing 20 mM $NaBH_4$ was performed at 4°C (6 M Alkali Soluble Solids, 6MASS). The 6 molar alkali extraction was performed using KOH instead of NaOH, because at this concentration KOH was found to improve extraction of xyloglucan, more than NaOH (*12*). The final unextracted material is called the residue (RES). After each extraction, solubilized polymers were separated from the insoluble residue by filtration on G2-glass filters. ChSS were dialyzed against 0.1 M NH_4OAc buffer at pH 5.2 prior to dialysis against demineralized water at 4˚C. DASS, 4MASS, 6MASS and RES were neutralized with 1 M HCl, dialyzed against demineralized water at 4˚C and freeze-dried.

2.2.3 Enzymatic fingerprinting

RSM fractions (in 10 mM NaOAc buffer pH 5.0, 5 mg/mL) were incubated with pure and well-characterized enzymes. The choice for specific enzymes to demonstrate the presence of specific polysaccharides was made based on the carbohydrate composition of the RSM

fractions. The enzymes used were polygalacturonase (*Aspergillus aculeatus*; 190 µg protein/mL (*13*), beta-galactosidase (*Aspergillus niger*; 15 µg protein/mL (Laboratory of Food Chemistry, unpublished data)), endo-galactanase (*Aspergillus niger*; 26 µg protein/mL, (*14*), endo-arabinanase (*Aspergillus aculeatus*; 7.3 mg protein/mL, (*15*), exo-arabinanase (*Chrysosporium lucknowense* C1 (*16*)), endo-mannanase (*Aspergillus niger*; 542 µg protein/mL (*17*)), a xyloglucan specific endo-glucanase (*Aspergillus aculeatus* (*18*)) and endo-xylanase I (*Aspergillus awamori*; 22 µg protein/mL (*19*)). Besides these pure enzymes, a commercial cellulase preparation (CellicCTec, Novozymes, Bagsvaerd, Denmark) was used. Enzymes were dosed at 0.5 µg enzyme-protein per 5 mg substrate.
The incubations were performed in 10 mM NaOAc buffer (pH 5.0) at 40˚C rotating 'head-over-tail' for 24 hours. All enzymes were inactivated by heating at 100˚C for 10 minutes. Digests were analyzed by HPSEC, HPAEC and MALDI-TOF MS, as described in section 2.2.4.

2.2.4 Analytical methods

Dry matter content was determined in duplicate by drying overnight in an oven at 103°C.
Protein content (Nx5.3 (*20*)) was determined in duplicate by the Dumas method (*21*) on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Interscience, Troy, NY, USA). Sample (~10 mg) was weighed into a cup and directly analyzed. D-methionine was used for calibration.
Lignin content was determined gravimetrically according to NREL in duplicate. After pre-hydrolysis with 72% (w/w) H_2SO_4 for 1 h at 30˚C, samples were hydrolyzed with 1 M H_2SO_4 at 100˚C for 3 h. Samples were filtered over G4 glass filters. The filtrate was measured for acid soluble lignin (ASL) spectrophotometrically at 205 nm. ASL was calculated according to the formula: ASL= (A * B * C)/(D * E), with A= absorption relative to 1 M H_2SO_4, B= dilution factor, C= filtrate volume, D= extinction coefficient of lignin (110 g L^{-1} cm^{-1}), and E= weight of substrate (g). The washed residue was dried (105°C, 18h), and weighed as acid insoluble lignin. Total lignin was calculated as the sum of acid soluble and acid insoluble lignin (*22*).
Neutral sugar composition was determined in duplicate according to Englyst and Cummings (*23*). After a pre-hydrolysis with 72% (w/w) H_2SO_4 for 1 h at 30°C, the samples were hydrolyzed with 1 M H_2SO_4 at 100°C for 3 h. The monosaccharides were derivatized to their alditol acetates and analyzed by gas chromatography (Focus-GC, Thermo Scientific, Waltham, MA, USA). Inositol was used as internal standard.

Uronic acid content was determined in duplicate according to the automated colorimetric m-hydroxydiphenyl assay (*24*), including tetraborate, using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration.

Acetyl- and methyl- ester content was determined in duplicate by High Performance Liquid Chromatography (HPLC) after treatment of the sample (20 mg/mL) with 0.4 M NaOH in isopropanol/water (1:1 (v/v)) on an Ultimate 3000 System (Dionex, Sunnyvale, CA, USA) equipped with an Aminex HPX-87H ion exclusion column (7.8mmx300mm; BioRad Laboratories, Hercules, CA, USA). The samples were eluted with 5 mM sulphuric acid at 40˚C and at a flow rate of 0.6 mL/min. Elution was followed by refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan). Quantification was performed using HPLC-grade acetic acid and methanol standards. Also, samples were analyzed without alkali addition in order to correct for presence of free acetic acid and methanol.

High Performance Size Exclusion Chromatography (HPSEC) was performed on an Ultimate 3000 System (Dionex) equipped with a set of four TSK-Gel superAW columns (Tosoh Bioscience, Tokyo, Japan) in series: guard column (6 mm ID x 40 mm) and separation columns 4000, 3000 and 2500 (6 mm ID x 150 mm). Samples (25 µL) were eluted with filtered aqueous 0.2 M sodium nitrate for 25 minutes at 40˚C and at a flow rate of 0.6 ml/min followed by refractive index detection (Shodex RI 101; Showa Denko K.K.). Calibration was performed using pullulan standards of 180, 738 Da and 6, 12, 23, 47, and 112 kDa (Sigma, St. Louis, MO, USA).

High Performance Anion Exchange Chromatography (HPAEC) was performed on an ICS-5000 System (Dionex) equipped with a CarboPac PA 1 column (2x250 mm), and Pulsed Amperiometric Detection. Elution was performed with a flow rate of 0.3 mL/min and a temperature of 40˚C. The elution profile used for quantification of di- and oligosaccharides was: 0-5 minutes isocratic 0.1 M NaOH, 5-15 minutes linear 0 to 0.1 M NaOAc in 0.1 M NaOH, 15-25 minutes linear 0.1 to 0.3 M NaOAc in 0.1 M NaOH, isocratic for 5 minutes at 1 M NaOAc in 0.1 M NaOH, followed by 20 minutes isocratic at 0.1M NaOH. WSS was ten times diluted before analysis The gradient used for analysis of enzyme digests was: 0-45 minutes linear from 0 to 0.7 M NaOAc in 0.1 M NaOH, isocratic for 5 minutes at 1 M NaOAc in 0.1 M NaOH, followed by 15 minutes isocratic at 0.1 M NaOH. Enzyme digests were ten times diluted before analysis.

Matrix Assisted Laser-induced Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) was performed using an UltraFlextreme workstation (Bruker Daltonics,

Bremen, Germany) equipped with a Smartbeam II laser of 355 nm and operated in the positive mode. After a delayed extraction time of 350 ns, the ions were accelerated to a kinetic energy of 22 kV and detected using a FlashDetector. The data were collected from averaging 200 laser shots, with the lowest laser energy needed to obtain sufficient spectra. External calibration was performed using maltodextrin (Paselli MD-6, AVEBE, Veendam, the Netherlands). Samples were desalted prior to analysis using AG 50W-X8 Resin (BioRad Laboratories, Hercules, CA, USA). 1 µL desalted sample was mixed with 1 µL matrix solution of 10 mg/mL 2,5-dihydroxy-benzoic acid (Bruker Daltonics) in 50% (v/v) acetonitrile and dried under a stream of air.

Glycosidic linkage analysis was performed as described elsewhere (*25*). Polysaccharides were methylated, followed by hydrolysis with 2 M TFA for 1 hour at 121°C and permethylated monomers were converted into their alditol acetates. Treatment with methyliodide was performed twice. The partially methylated alditol acetates were identified by GC-MS using a Trace GC coupled to a DSQ-II (both Thermo Scientific) equipped with a Restek RTX-35MS column (Restek Corporation, Bellefonte, PA, USA). A temperature gradient was applied from 120°C to 250°C in 52 minutes, proceeded by a hold time of 5 minutes at 250°C. MS detection of masses 50-450 m/z was performed.

2.3 Results and discussion

2.3.1 General composition of RSM

Rapeseed meal (RSM) was analyzed for its carbohydrate, protein and lignin contents (Table 2.1). Protein (Nx5.3; 30% w/w) and lignin contents (24% w/w) of our *B. napus* meal, were similar to *B. campestris* meal, another rapeseed cultivar published previously (*26, 27*). The carbohydrate content (36% w/w) was higher compared to the 28% (w/w) found for *B. campestris* meal (*6*). The carbohydrate composition of RSM is presented in Table 2.2. RSM was high in glucosyl (40 mol%), arabinosyl (19 mol%) and uronyl residues (15 mol%). Compared to *B. campestris* meal (*6*), in *B. napus* meal only about half of the arabinosyl and galactosyl residues were present, but twice as much glucosyl residues were found. The content of uronyl residues was the same.

Table 2.1. Composition of rapeseed meal (RSM) and its fractions (%w/w). RSM: Rapeseed meal, WSS: Water Soluble Solids, WUS: Water Unextractable Solids, ChSS: Chelating Agent Soluble Solids, DASS: Dilute Alkali Soluble Solids, 4MASS: 4 Molar Alkali Soluble Solids, 6MASS: 6 Molar Alkali Soluble Solids, RES: Residue.

	RSM	WSS	WUS	ChSS	DASS	4 MASS	6 MASS	RES
Yield [a]	100 *(100)*	26 *(27)*	64 *(59)*	6 *(3)*	4 *(2)*	9 *(5)*	10 *(18)*	31 *(39)*
Total sugar [b]	36	37	33	16	14	17	60	41
Protein *(Nx5.3)* [b]	30	14	34	41	44	33	18	23
Lignin (acid soluble) [b]	24 (10)	n.a.[c]	22 (9)	n.a.	n.a.	n.a.	n.a.	23 (6)
Acetyl esters [b]	0.26	0.26	0.55	0.11	0	n.a.	n.a.	n.a.
Methyl esters [b]	0.15	0.49	0.18	0.50	0.35	n.a.	n.a.	n.a.

[a] Expressed as g/100g RSM, as g/100g total carbohydrate between brackets. [b] Expressed as %w/w. [c] Not analysed.

2.3.2 Water soluble fraction of RSM

When extracted with hot water (70°C), 26% of the dry matter and 27% of the total carbohydrate content of RSM was solubilized (Table 2.1). The water soluble fraction (WSS) mainly contained glucosyl (64 mol%) and some galactosyl residues (17 mol%). HPAEC analysis showed that this WSS fraction mainly contained small saccharides, like fructose, sucrose, raffinose and stachyose, which contributed to the total carbohydrate content of the WSS for 4, 50, 3 and 18%, respectively. These values are in agreement with previously published data for *B. campestris* (*5, 28*).

For *B. campestris* meal, besides small saccharides also a water-soluble arabinan (*29*) and an acidic arabinogalactan (*8*) have been reported. In our *B. napus* meal 6% (w/w) of total carbohydrate in WSS was arabinosyl and 5% (w/w) uronyl residues, but their polymeric structure was not further investigated.

2.3.3 Sequential extraction of WUS

The water unextractable solids (WUS) fraction was composed of 33% carbohydrates, mainly glucosyl (32 mol%), arabinosyl (25 mol%), uronyl (18 mol%) and xylosyl residues (12 mol%) (Table 2.2). Of the uronic acids residues 35% was substituted with methanol groups and 36% with acetic acid groups. In order to investigate the polysaccharide structures in detail, the water unextractable solids (WUS) were sequentially extracted with chelating agent (ChSS) to release calcium-bound pectins, dilute alkali (DASS) to release pectins tightly bound to hemicellulose, and 4 and 6 molar alkali (4 MASS and 6 MASS) to release hemicelluloses. Cellulose will remain in the residue (RES).

The yields and general composition of the fractions are shown in Table 2.1, while the detailed carbohydrate composition of the fractions is presented in Table 2.2. Fructose and fructose-containing oligosaccharides influence the carbohydrate composition and yields, since they are not correctly analyzed using the alditol acetate method. ChSS and DASS yields were relatively low, which was unexpected. Proteins were gradually extracted, which resulted in fractions having a quite high protein content (14-44% w/w). It has to be noted that the protein content of the ChSS fraction is also influenced by the presence of residual CDTA in this fraction. Based on dry matter, the residue after extraction of RSM was the largest fraction, which is mainly due to the recovery of lignin in the residue (23%

w/w). This is 30% of the lignin present in the RSM, meaning the rest of the lignin is co-extracted in the alkali fractions.

The mass balance of the extraction (Table 2.1 and 2.2), showed a recovery of 86% of the dry matter of RSM. Based on the total carbohydrate content, 94% could be recovered, of which recovery of each individual constituent monosaccharides was in the range of 78-98% (Table 2.2). From *Brassica campestris* meal less material could be recovered during the extraction procedures reported (*5, 6*), which expectedly resulted in less representative fractions.

Molecular weight distribution of the polysaccharides in extracted RSM fractions was analyzed by HPSEC (Supplemental figure 2.1). Different populations of polysaccharides were distinguished. The DASS fraction contained polysaccharides with a molecular mass of around 14 kDa, as based on pullulan standards, and some smaller saccharides of 1.6 and 4 kDa. The 4 MASS fraction contained polysaccharides of around 25 and 350 kDa and smaller saccharides of 2.3 kDa. The 6 MASS fraction contained even larger polysaccharides of around 440 kDa and also (poly)saccharides of 2.3 and 25 kDa as also found in the 4 MASS fraction. ChSS is not shown, since CDTA is difficult to remove from pectins (*30*), thereby interfering with the HPSEC-analysis.

2.3.4 Sugar composition of RSM fractions

The ChSS fraction, representing 3% of total carbohydrate of RSM, was high in uronyl residues (71 mol%), most likely present as homogalacturonan (Table 2.2). Of these uronic acid residues 60% was substituted with methanol esters and 9% with acetic acid esters. Next to uronyl residues, arabinosyl residues were abundant (14 mol%), which is expected to be present as arabinan side chains, as often reported for pectin structures (*3*).

Table 2.2. Molar sugar composition and sugar yields of rapeseed meal (RSM) and its fractions (RSM: Rapeseed meal, WSS: Water Soluble Solids, WUS: Water Unextractable Solids, ChSS: Chelating Agent Soluble Solids, DASS: Dilute Alkali Soluble Solids, 4 MASS: 4 Molar Alkali Soluble Solids, 6 MASS: 6 Molar Alkali Soluble Solids, RES: Residue).

	Molar composition [a]							Yield [b]						
	Rha	Ara	Xyl	Man	Gal	Glc	UA	Rha	Ara	Xyl	Man	Gal	Glc	UA
RSM	2	19	8	6	10	40	15	100	100	100	100	100	100	100
WSS	tr [c]	7	1	7	17	64	5	0	9	4	31	43	42	8
WUS	1	25	12	4	8	32	18							
ChSS	2	15	4	2	4	3	71	3	2	1	1	1	0	12
DASS	tr	44	6	3	10	9	29	0	4	1	1	2	0	3
4 MASS	tr	17	20	15	13	30	5	0	4	11	12	6	3	2
6 MASS	1	29	22	2	13	23	11	13	26	48	6	22	10	12
RES	4	20	6	4	6	40	20	81	36	28	27	24	36	46
Recovery								97	81	93	78	98	91	83

[a] Expressed as anhydro-units: Ara: Arabinose, Xyl: Xylose, Man: Mannose, Gal: Galactose, Glc: Glucose, UA: Uronic acids.
[b] Expressed as g/100g monosaccharide unit. [c] Trace.

The DASS fraction, representing 2% of total carbohydrate content of RSM, was high in arabinosyl (44 mol%) residues, probably originating from arabinan side chains of pectin present (Table 2.2). The latter was indicated by the presence of 29 mol% uronyl residues.
The 4 MASS fraction, representing 4% of total carbohydrate content of RSM, contained quite some xylosyl (20 mol%) and glucosyl (30 mol%) residues, which is expected to be present as xyloglucan (*31*). Also arabinosyl (17 mol%), mannosyl (15 mol%) and galactosyl (13 mol%) residues were present. This could indicate the presence of arabinan, (galacto-)mannan and (arabino-)galactan.
The 6 MASS fraction, representing 17% of total carbohydrate content of RSM, consisted of arabinosyl (29 mol%), xylosyl (22 mol%) and glucosyl residues (23 mol%), which could indicate the presence of arabinan, arabinoxylan and/or xyloglucan. Also, galactosyl (12 mol%) and uronyl residues (11 mol%) were present.
The residue, representing 39% of total carbohydrate content of RSM, contained mainly (40 mol%) glucosyl residues and also quite some arabinosyl and uronyl residues (each 20 mol%). The latter indicates that are still (pectic) polysaccharides tightly associated with cellulose microfibrils (*18*). This lead to the assumption that
the cell wall polysaccharide matrix of rapeseed meal is very strongly associated. A similar conclusion has been proposed for *B. campestris* meal (*6*).
More detailed analysis is necessary to analyze the polymeric structure of the extracted cell wall polysaccharides from RSM. This is studied using linkage type analysis, which will give information on the type of carbohydrate linkages present between the constituent monosaccharides (Section 2.3.5) and using pure enzymes, which will be helpful in elucidating the presence of specific polysaccharides (Section 2.3.6).

2.3.5 Glycosidic linkage type of RSM fractions

Data obtained from linkage type analysis of the RSM fractions (Table 2.3) can only be used in a qualitative way instead of quantitative due to the poor DMSO solubility and the high amount of uronic acids, which are not detected in this method, present of some samples (*32*).
Arabinosyl residues in the DASS and 4MASS fraction were mainly present as terminal residues (51% of the arabinosyl residues present) or linked via 1,2,5-bonds (36% of the arabinosyl residues) (Table 2.3). This indicates the presence of an arabinan with branches at the O-2 position. This is different from *B. campestris* meal, for which a highly branched

arabinan was reported with branching at both the O-2 and O-3 position. The arabinosyl residues in the 6MASS fraction were mainly present as terminal units and linear 1,5-linked-arabinosyl residues. The arabinan present in this fraction is much more linear than the arabinan present in the DASS and 4MASS fraction. The linearity of the arabinan could be responsible for the fact that these arabinan structures were more difficult to extract (*33*).

Table 2.3. Sugar linkage composition of rapeseed meal (RSM) fractions; expressed as anhydro-units: Ara: Arabinose, Xyl: Xylose, Man: Mannose, Gal: Galactose, Glc: Glucose, UA: Uronic acids (mol%).

	WUS	DASS	4 MASS	6 MASS
t-Ara[a]	18	41	16	15
1,2-Ara		1	1	
1,5-Ara	10	9	2	11
1,2,5-Ara	6	29	10	4
Total Ara	**34**	**80**	**29**	**30**
t-Xyl	9	3	7	12
1,2-Xyl	2	1	10	3
1,4-Xyl	3	1	3	7
Total Xyl	**14**	**5**	**20**	**22**
1,4,6-Man			3	1
Total Man			**3**	**1**
t-Fuc	2	1	2	5
1,2,4-Fuc	7	3		8
Total Fuc	**9**	**4**	**2**	**13**
t-Gal	3		4	6
1,2-Gal			5	7
1,3-Gal			7	
1,3,6-Gal		7	4	
Total Gal	**3**	**7**	**20**	**13**
1,4-Glc	25	5	17	6
1,4,6-Glc	16		11	15
Total Glc	**41**	**5**	**28**	**21**
T/B [b]	**1.10**	**1.15**	**1.04**	**1.36**

[a] t: terminal [b] T/B: ratio terminally linked residues: branching points

Besides arabinosyl residues, the 4MASS fraction contains galactosyl residues, which were mainly 1,3-linked. Most likely, our *B. napus* meal contained arabinogalactan type II, since also some terminal and 1,3,6-linked galactosyl residues were found. Type II arabinogalactan was also found in *B. campestris* meal (*8*). Glucosyl residues in the 4MASS fraction were mainly 1,4- and 1,4,6-linked, and xylosyl was present as terminal residues

and 1,2-linked, as was also described for the xyloglucan found in *B. campestris* meal (*7*). The xyloglucan in *B. napus* meal is also fucosylated, similar to *B. campestris* meal (*10*). Around 15% of the xylosyl residues were 1,4-linked, which means that besides xyloglucan, a xylan is present.

2.3.6 Enzymatic fingerprinting of RSM fractions

The ChSS fraction could not be analyzed by linkage type analysis, due to the high content of uronyl residues, which are not detected is this in this method. However, enzymatic fingerprinting was successful in characterizing the polysaccharides present. After incubation of the ChSS fraction with polygalacturonase, a range of galacturonic acid-oligosaccharides of DP 1-8 were formed (Figure 2.1), originating from homogalacturonan structural elements.

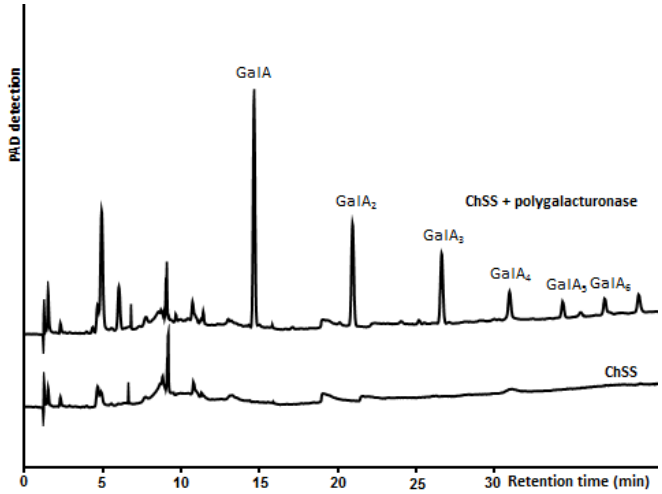

Figure 2.1. HPAEC chromatogram of RSM ChSS before and after incubation with polygalacturonase. GalA: galacturonic acid; $GalA_n$: linear galacturonic acid-oligosaccharides of DP_n (n=2-6).

In addition, after incubation of ChSS fraction with endo-arabinanase, arabinose oligomers of DP 2-4 were produced (data not shown), indicating the presence of an arabinan. This is in line with results from linkage type analysis (Section 3.5).

Incubation of the DASS fraction with an endo-arabinanase did not show any degradation products, but when an exo-arabinanase was added arabinose and arabinobiose were formed (Figure 2.2). This confirms the result from linkage type analysis that DASS

contained a branched arabinan. Next, a combination of beta-galactosidase and endo-galactanase showed formation of galactose and galactobiose. This could indicate that there is arabinogalactan type I, having β-1,4-linkages, or galactomannan present as also described for *B. campestris* meal previously (*8*). However, in the linkage type analysis, only 1,3,6-linked galactosyl residues were analyzed in the DASS fraction, no 1,4 linkage was found.

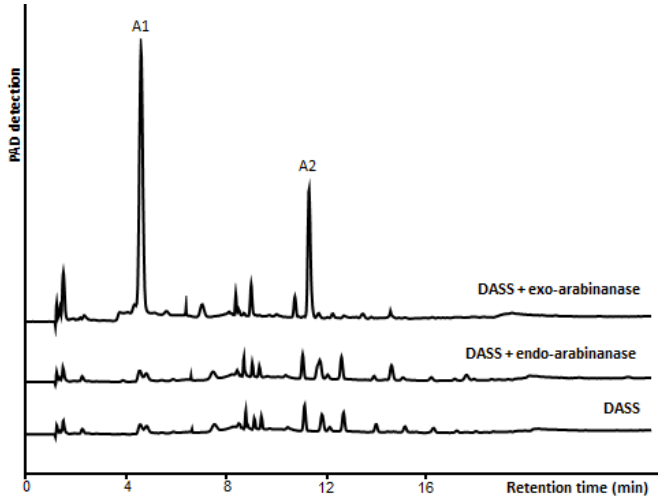

Figure 2.2. HPAEC chromatogram of RSM DASS before and after incubation with endo- and exo-arabinanase (A1= arabinose, A2= arabinobiose).

Simultaneous incubation of the 4MASS fraction with β-galactosidase and endo-galactanase resulted in hexose-oligomers up to DP 6, analyzed with MALDI-TOF-MS. After mannanase incubation, hexose-oligosaccharides up to DP 7 were formed. Due to the known specificity of the enzymes used, it can be said that these hexose oligomers formed were built from galactosyl and mannosyl residues. This indicates the presence of galactomannan. When the 4 MASS fraction was incubated with a xyloglucan-specific glucanase, typical xyloglucan oligomers were formed (Figure 2.3A). It is known that only part of the polysaccharide fraction can be degraded by this enzyme (*18*). From the part that is degraded, XSGG, LSGG (both XXGG-type), XXFG and XLFG (both XXXG-type) were formed

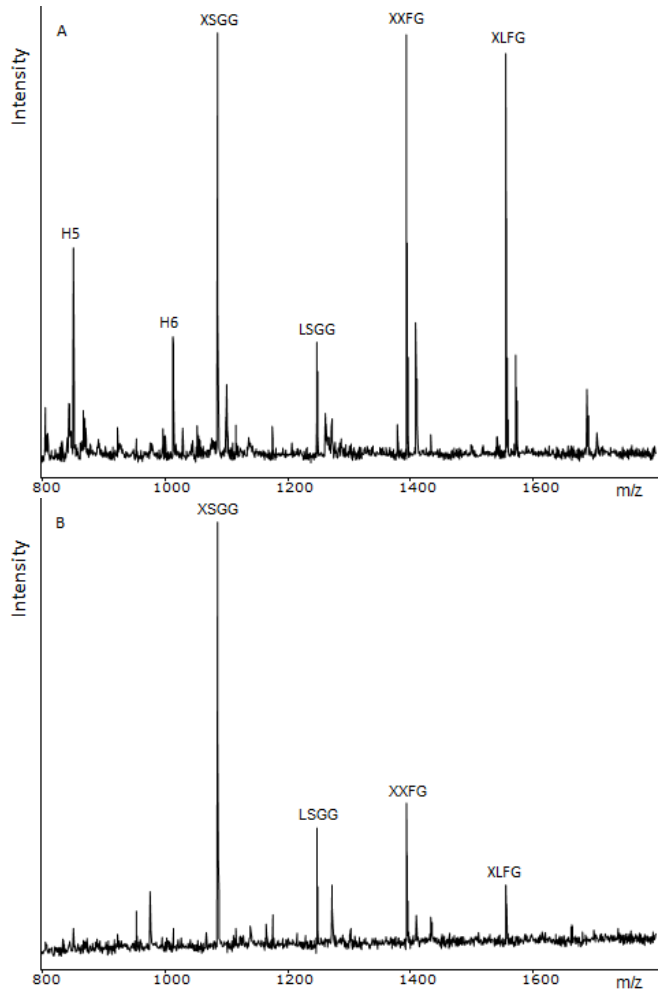

Figure 2.3. MALDI-TOF-MS spectrum of (A) RSM 4MASS incubated with a xyloglucan-specific glucanase and (B) RSM RES incubated with a commercial cellulase preparation. H= hexose; nomenclature of xyloglucan-oligomers according to Fry et al. (*34*).

(based on nomenclature described by Fry et al. (*34*)). This indicated the presence of galactosyl and fucosyl decorations on the xylosyl residues. The XXFG and XLFG moieties have also been reported for the 4MASS fraction of *B. campestris* meal (*6*). Siddiqui & Wood (*10*) reported the presence of 1,4,6-linked glucosyl and 1,4-linked glucosyl in a molar ratio of 3:1. In our view, this can be attributed to the presence of XXXG-type xyloglucan. From a phylogenetic point of view the XXXG-type structures are expected,

while the XXGG-type are not. The fact that in *B. napus* meal both the XXGG- and XXXG-type xyloglucan are found is not common, although it has been described before in the Poaceae family for rice (*35*).

When the 4 MASS fraction was incubated with an endo-xylanase, pentose oligomers up to DP 5 with an O-methylated uronic acid attached were formed. Such glucuronoxylan oligomeric structures have been reported before for *B. campestris* meal (*6*).

Incubation of the 6MASS fraction with endo-xylanase revealed the formation of pentose-oligomers up to DP 6 with O-methylated uronic acid attached (data not shown), comparable to the 4MASS fraction. Besides xylosyl and uronyl residues, also arabinosyl, galactosyl and glucosyl residues were found (Table 2.2). Therefore this fraction was also incubated with xyloglucan-specific glucanase, endo-arabinanase, and the combination of endo-galactanase and beta-galactosidase. However, all incubations did not show release of oligosaccharides. This indicates that the structure of carbohydrates extracted in the 6MASS fraction was too complex to be degraded by the enzymes used.

Degradation of the RES fraction with a commercial cellulase preparation confirmed the presence of hexose oligomers (from cellulose) next to xyloglucan-oligomers (Figure 2.3B). The xyloglucan-oligosaccharides confirmed that the xyloglucan in the residue consisted of building blocks of the XXGG-type and XXXG-type decorated with galactosyl, arabinosyl and fucosyl residues. When the residue

was incubated with a xyloglucan-specific glucanase, no degradation was observed (data not shown), as was expected because xyloglucan and cellulose can be tightly bound by hydrogen bonding (*18*).

Next to cellulose and xyloglucan, the residue also contained quite some rhamnosyl, arabinosyl and uronyl residues, probably originating from pectic polysaccharides. A cellulose-arabinan complex (*36*) and a cellulose-rhamnogalacturonan (*37*) have both been described before.

2.4 Conclusions

The comparison of polysaccharides present in *B. napus* and *B. campestris* meal is presented in Table 2.4. Although both meals belong to the same phylogenetic family of Brasicaceae and similar polysaccharides were found, also distinct differences in cell wall polysaccharide structures are present. *B. napus* contained arabinan with only O-2 branches (instead of branching at O-2 and O-3) and XXGG-type xyloglucan (besides XXXG-

type xyloglucan), which were not found in *B. campestris*. Homogalacturonan, arabinogalactan and glucuronoxylan are similar in structure.

In the residue after sequential extraction, besides cellulose, still pectic polysaccharides were found. This implies that RSM has a rigid matrix and therefore different pre-treatments have to be explored to improve accessibility of the cell wall polysaccharides and to increase digestibility for monogastric animals.

Table 2.4. Summary of polysaccharides present in *B. napus* meal (this paper) and *B. campestris* meal (*5*).

	***B. napus* meal**	***B. campestris* meal**
1,5-linked arabinan branched at O-2	+	+
1,5-linked arabinan branched at O-3		+
Galactomannan	+	
Homogalacturonan	+	+
Rhamnogalacturonan I	+	
Type I arabinogalactan		
Type II arabinogalactan	+	+
Glucuronoxylan	+	+
XXGG-type xyloglucan	+	
XXXG-type xyloglucan	+	+

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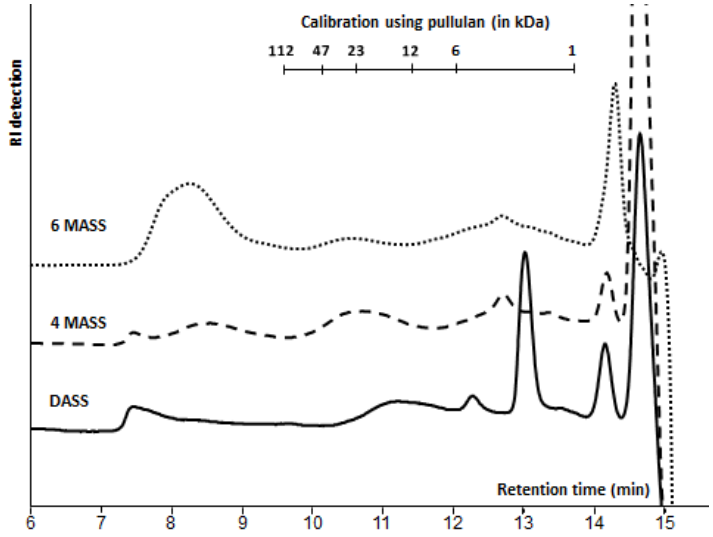
Supplemental figure



Supplemental figure 2.1. HPSEC elution patterns of DASS (solid line), 4 MASS (striped line) and 6 MASS (dotted line).

Chapter 3

Residual carbohydrates from *in vitro* digested processed rapeseed (*Brassica napus*) meal

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Abstract

Rapeseed meal (RSM) was subjected to different physical or chemical pre-treatments to decrease residual, hard to degrade carbohydrates and to improve fermentability of RSM polysaccharides. Next, these pre-treated samples were *in vitro* digested and fermented, with or without the addition of commercial pectinolytic enzymes. Remaining carbohydrates were quantified, and two physical characteristics were analyzed: (1) water-binding capacity (WBC) of the insoluble residue and (2) viscosity of the soluble fraction. Mild acid pre-treatment in combination with commercial pectinolytic enzyme mixtures showed best digestion of RSM carbohydrates; only 32% of the total carbohydrate content remained. For most pre-treatments, addition of commercial pectinolytic enzymes had the strongest effect on lowering the WBC of the *in vitro* incubated RSM. In the cases that less carbohydrate remained after *in vitro* digestion, the WBC of the residue decreased, and less gas seems to be produced during fermentation.

3.1 Introduction

Rapeseed meal (RSM) is a by-product from the production of rapeseed oil, which is used as an edible oil as well as for biodiesel. After solvent extraction of the oil, the meal is dried by solvent-toasting and used as animal feed for its high protein content. RSM has an energy value (net energy/8.8) of 0.71 MJ/ kg for pigs (*1*). The high demands for biofuels led to an increase in the production of biodiesel, and therefore more RSM will become available for the animal feed industry. Apart from protein, RSM is rich in non-starch polysaccharides (NSP), which potentially could be used as an energy source in animal feed. Monogastric animals lack the necessary digestive enzymes that can degrade NSP. Still, in the large intestine (and already in the small intestine of pigs) these carbohydrates are fermented into short-chain fatty acids, gases, and microbial biomass (*2*). Pectins are the main carbohydrates present in dehulled rapeseed (*3*). Pectins are a very diverse group of polysaccharides, consisting of homogalacturonan, rhamnogalacturonan I and II, and xylogalacturonan. Along with pectins, cellulose and hemicelluloses (like xyloglucan) are present. Apart from polysaccharides RSM also contains sucrose and oligosaccharides, such as raffinose and stachyose (*4*). An option to increase the energy value of RSM is to stimulate the degradability of polysaccharides for (monogastric) animals by pre-treatment of the RSM before inclusion into the diet. Hereto, different techniques such as fine milling, extrusion, chemical pre-treatment, and enzyme addition are good candidates. Milling increases the surface area of a product. Shear is used to increase the extractability of soluble carbohydrates and the accessibility for (digestive) enzymes. During extrusion the product is exposed to a high temperature (90−160 °C) for a short time (30−120 s) at high pressures and at relatively low moisture contents (<30%) (*5*). Chemical pre-treatment with sulfuric acid has been the traditional method to increase extractability of carbohydrates from lignocellulosic material. Recently, also dicarboxylic organic acids at elevated temperatures have been shown to open up the cell wall and to increase carbohydrate solubility (*6*). These could be more suitable in animal nutrition than the use of sulfuric acid, because no additional sulfates will be introduced in the diet.

Apart from the above-described pre-treatments, addition of NSP-degrading enzymes is an option to open up the cell wall material and make it more accessible for digestive enzymes. Along with polysaccharide degradation, they can reduce digesta viscosity and detrimental effects on digestion and absorption (*7*). So far, RSM digestion studies have focused on protein digestibility (*8, 9*). or when focused on polysaccharide digestibility,

only neutral and acid detergent fiber content were analyzed (*10*). *In vitro* digestibility studies of other oil seeds, for example, sunflower meal and soybean meal, have been performed. Addition of commercial enzymes was shown to increase total carbohydrate solubilization and decreased viscosity (*11*); however, constituent glycosyl residues were not analyzed. The aim of the present study is to improve the *in vitro* digestibility of RSM and to characterize residual carbohydrates and their constituent glycosyl residues. Especially the combination of pre-treatment and the addition of commercial pectinolytic enzymes are hypothesized to have significant effects on carbohydrate digestion. Digestibility is evaluated by an *in vitro* digestion and fermentation study. The changes in carbohydrate composition and molecular weight distribution are analyzed and related to water-binding capacity (WBC) and viscosity, respectively, which are also important aspects for the transit in the gastrointestinal tract.

3.2 Materials and methods

3.2.1 Plant material

Rapeseed meal (90% w/w dry matter) was supplied by Nutreco (Boxmeer, The Netherlands).

3.2.2 Treatments

The effects of technological pre-treatment and commercial pectinolytic enzyme addition were studied. Different technologies and accompanying settings, such as temperature, flow, concentration, and residence time, were tested in pre-experiments, with the criterion being the optimal solubilization of NSP from rapeseed meal.

Wet-milling was performed using a laboratory-scale refiner (Sprout- Waldron, Muncy, PA, USA) with a feed rate of 150 kg/h, a water flow rate of 480 L/h at 3000 rpm, and a distance of 0.07 mm between the disks. The temperature of the processed product was around 36 °C. Samples were freeze-dried before analysis.

Extrusion was performed with a double-screw extruder (Baker-Perkins Ltd., Peterborough, UK) at 120 °C (product temperature = 119 °C), 250 rpm screw speed, and die size = 6 mm at 20% (w/w) moisture.

Autoclave pre-treatment was performed using a Varioklav 25T tabletop (Thermo Scientific, Waltham, MA, USA) at 120 °C for 30 min at 20% (w/w) moisture.

Mild acid pre-treatment was performed after the samples had been soaked in 11 mM (or 1.4% w/w acid based on dry matter of the substrate) maleic acid at a concentration of 100 g/L. Samples were heated in the above-described autoclave for 30 min at 130 °C. The final pH of the solution after heat treatment was 5.2. Samples were freeze-dried before analysis. Commercial enzyme mixtures Pectinex UltraSP (Novozymes, Bagsvaerd, Denmark) and Multifect Pectinase FE (Genencor, Rochester, NY, USA) were, if appropriate, added (250 µL per 10 g of substrate for each enzyme mixture) in the first step of the *in vitro* digestion.

3.2.3 In vitro *digestion and fermentation*

In vitro digestion was performed according to the method of Boisen and Fernandez (*12*) as modified by Sappok et al. (*13*) without further milling. Briefly, RSM was incubated in duplicate with pepsin at pH 3.5 at 40 °C for 75 min, followed by the addition of pancreatin and amyloglucosidase and incubation at pH 6.8 at 40 °C for 3.5 h. The final residues were washed with demineralized water, centrifuged (twice 10 min at room temperature and 3030g), decanted, freeze-dried, and used for analyses. Also, the corresponding supernatants were collected and kept frozen for further analyses of the soluble fraction.

Residues were used as a substrate for *in vitro* fermentation according to the method of Williams et al. (*14*) using fecal inocula from sows receiving commercial diets containing the NSP sources 30% barley, 20% wheat middlings, 10% maize, 7.5% rapeseed meal, 5% wheat, 5% soy hulls, and 1.5% linseed. Besides this, the diet contained synthetic amino acids, salts, and oil. Samples were incubated at pH 6.8 at 40 °C for 72 h, and cumulative gas production was measured in time with the fully automated time-related gas production system (*15*).

3.2.4 Analytical methods

The geometric mean diameter (GMD) of untreated RSM was analyzed using the wet sieve method and calculated according to the ASAE method (*16*). The GMD of pre-treated RSM was analyzed on a CoulterCounter (Beckman Coulter, Brea, CA, USA).

Protein content (N × 5.3) was determined according to the Dumas method of the AOAC (*17*) on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Interscience, Troy, NY, USA). Sample (~10 mg) was weighed into a cup and directly analyzed. D-Methionine was used for calibration.

Total starch content was determined enzymatically using the total starch assay procedure K-TSTA 04/2009 (Megazyme, Bray, Ireland).

Neutral sugar composition was determined by gas chromatography according to the method of Englyst and Cummings (*18*). After a pre-treatment with 72% (w/w) H_2SO_4 for 1 h at 30 °C, the samples were hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. Afterward, the constituent glycosyl residues were derivatized into alditol acetates and analyzed using a GC (Focus-GC, Thermo Scientific, Waltham, MA, USA). Inositol was used as internal standard.

Uronic acid content was determined according to the automated colorimetric m-hydroxydiphenyl assay (*19*), including tetraborate, using an auto-analyzer (Skalar Analytical, Breda, The Netherlands). Galacturonic acid was used for calibration.

Lignin content was determined gravimetrically. After a pre-treatment with 72% (w/w) H_2SO_4 for 1 h at 30 °C, samples were hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. Samples were filtered over a G4 glass filter (Schott Duran). The acid-insoluble residue was dried (105 °C, 18 h) and weighed.

Water-binding capacity (WBC) was determined by soaking 250 mg of raw material or freeze-dried residue in 10 mL of water for 24 h at room temperature. Samples were centrifuged at 3274g for 20 min at room temperature and subsequently drained inverted for 15 min. WBC was calculated as grams of water held per gram of dry material.

Viscosity of the soluble fraction was determined with a rheometer (RheoLab QC, Anton Paar GmbH, Graz, Austria) with a double-gap module attached. Prior to measurement, samples were freeze-dried and solubilized in water to 1/10 of the original volume. Viscosity was measured at 40 °C and a shear rate of 122 s−1, mimicking the gastrointestinal tract (*20*).

High-performance size exclusion chromatography (HPSEC) was performed on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a set of four TSK-Gel superAW columns (Tosoh Bioscience, Tokyo, Japan) in series: guard column (6 mm i.d. × 40 mm) and separation columns 4000, 3000, and 2500 (6 mm i.d. × 150 mm). Samples (25 µL) were eluted with filtered aqueous 0.2 M sodium nitrate at 40 °C at a flow rate of 0.6 mL/min followed by refractive index detection (Shodex RI 101; Showa Denko K.K., Kawasaki, Japan). Prior to analysis, samples were freeze-dried and solubilized in water to 1/10 of the original volume. Calibration was performed using pullulan standards of 180 and 738 Da and 6, 12, 23, 47, and 112 kDa (Sigma, St. Louis, MO, USA).

From the HPSEC elution, fractions were collected using a Gilson FC204 fraction collector (Gilson Inc., Middleton, WI, USA), collecting 0.42 min per well. Of the fractions of interest, sugar composition was determined after drying at 40 °C under a stream of nitrogen gas. Samples were analyzed by methanolic HCl/TFA hydrolysis, by first solvolyzing them in 2 M HCl in dry methanol at 80 °C for 16 h, followed by hydrolysis with 2 M TFA at 121 °C for 1 h (*21*). The monomers were analyzed by high-performance anion exchange chromatography (HPAEC), and postcolumn addition was performed on an ICS-3000 unit (Dionex, Sunnyvale, CA, USA) using a CarboPac PA1 column (2 × 250 mm). Samples (25 µL) were eluted isocratically in 30 min with Millipore water. Afterwards, the following elution profile was applied: 30−45 min, 0.1 M NaOH−0.4 M NaOAc in 0.1 M NaOH; 45−50 min, 1 M NaOAc in 0.1 M NaOH; 50−58 min, 0.1 M NaOH; 58−73 min, Millipore water (equilibration). A flow of 0.1 mL/ min 0.5 M NaOH was added post column allowing pulsed amperiometric detection.

3.2.5 Calculations and statistical analysis

Calculations on in vitro fermentability. Data of cumulative gas production (mL/g DM) for each bottle were modeled according to the monophasic model described by Groot et al. (*22*).

$$G = \frac{A}{1+(\frac{B}{t})^C}$$

where G is the total gas production (mL/g DM), A the asymptotic gas production (mL/g DM), B the time at which half of the asymptotic gas production has been reached (h), C the switching characteristic of the curve, and t the time (h).

Statistical analysis. In a 5 × 2 factorial arrangement, the effects of technological pre-treatment (T) and commercial pectinolytic enzyme addition (E) were studied. The results from the *in vitro* experiment were statistically analyzed using the General Linear Models procedure with a SAS program (SAS Institute, version 9.2). The model used to describe the data was: $Yij = \mu + Ti + Ej + Ti \times Ej + \varepsilon ij$

where Yij is the response variable, µ the overall mean, Ti the technological pre-treatment (i = untreated, wet mill, autoclave, extruder, or mild acid pre-treatment), Ej the addition of commercial pectinolytic enzymes (j = yes or no), Ti × Ej the interaction between technological pre-treatment and enzyme addition, and εij the error term. Residuals were

tested for normality, using the Shapiro−Wilk test. A posthoc test was performed using multiple comparisons with Tukey adjustment.

3.3 Results and discussion

3.3.1 Characteristics of RSM

Industrial RSM (Table 3.1; untreated) is mainly composed of carbohydrates (48%), protein (33%), lignin (13%), fat (3%), and ash (3%). Apart from cellulose, the sugar composition (Table 3.1) indicated the presence of homogalacturonan, arabinan, and minor amounts of xyloglucan and arabinogalactan, which was expected (*3, 23*). Wet milling and mild acid pre-treatment decreased the total sugar content slightly, from 48% in untreated RSM to 45 and 43% w/w DM, respectively. Only small changes in sugar composition were noted, so all glycosyl residues were assumed to be stable during processing. The starch content was very low (<1% w/w DM) for all samples. The particle size distribution was affected by the various types of pre-treatment (Table 3.1). The untreated RSM used in this study had a GMD of 520 μm; autoclave and mild acid pre-treatment were similar. After extrusion and wet-milling, the GMD decreased to 343 and 135 μm, respectively. Particle size reduction is known to increase digestibility in pigs of dry matter and NSP in barley (*24*) and dried distillers grains with solubles (DDGS) (*25*).

The WBC was affected by processing (Table 3.1). Without processing, 1 g of untreated RSM could hold 2.4 g of water. After wet-milling, extrusion, and mild acid pre-treatment, the WBC increased to 3.5, 3.9, and 4.8 g water/g RSM, respectively. During milling, the surface area of the product increases, potentially increasing the water-holding capacity as reported for, for example, peas (*26*). During extrusion, the cell wall is opened up by heat and shear, creating pores in which water can be trapped (*27*). During mild acid pre-treatment, the lower pH probably degrades acid labile pectin structural elements, thereby increasing the WBC even more.

Table 3.1. Composition, particle size, and water-binding capacity of rapeseed meal, before and after treatments.

	Untreated	*Acid*	*Autoclave*	*Extruder*	*Wet mill*
Protein [a]	33	29	29	26	30
Total carbohydrates [a]	48	43	48	50	45
(of which starch [a])	(1)	(1)	(1)	(1)	(1)
Lignin [b]	13	n.a.[c]	n.a.	n.a.	n.a.
Molar composition of carbohydrates [d]					
Rha	0	1	1	0	1
Ara	13	14	13	13	14
Xyl	6	7	6	5	6
Man	6	5	4	12	9
Gal	7	8	7	7	7
Glc	28	29	27	21	24
UA	40	38	43	42	40
Particle size (µm)	520	499	520	343	135
WBC [e] (g water/g material)	2.4	4.8	2.4	3.9	3.5

[a] g/100g of dry matter. [b] acid insoluble lignin. [c] not analyzed. [d] mol%; presented as anhydrosugar moieties, Rha= rhamnose, Ara= arabinose, Xyl= xylose, Man= mannose, Gal= galactose, Glc= glucose, UA= uronic acid. [e] WBC= Water Binding Capacity.

3.3.2 In vitro *incubation*

For the first time, this paper presents a detailed study on residual carbohydrates and their constituent glycosyl residues after *in vitro* digestion of RSM. Solubles were separated from the residues after *in vitro* digestion. Residues were analyzed separately, because the carbohydrate structures that cannot be digested are of interest and it is known that soluble carbohydrates are more easily digested and fermented *in vivo* than insoluble carbohydrates (*28*). However, the *in vivo* part of the solubilized carbohydrates will still be available for fermentation. After *in vitro* digestion, 45−50% of total NSP remained for (un)treated RSM. When looking at the untreated RSM after *in vitro* incubation, nearly all of the arabinosyl (19% w/w of total sugar) and xylosyl (8% w/w) residues remained in the residue, whereas up to 60% of the glucosyl and uronyl residues are digested. Between pre-treatments no large differences were seen (Figure 3.1). When commercial pectinolytic enzymes were added after wet-milling and extrusion, remaining NSP decreased to 36% and for mild acid pre-treatment even to 32%. This indicates that the NSP fraction becomes more accessible for commercial pectinolytic enzymes after these two pre-treatments. The

interaction between thermal pre-treatment and addition of commercial pectinolytic enzymes has previously been found for apparent ileal digestibility of pre-treated soybean (*8*).

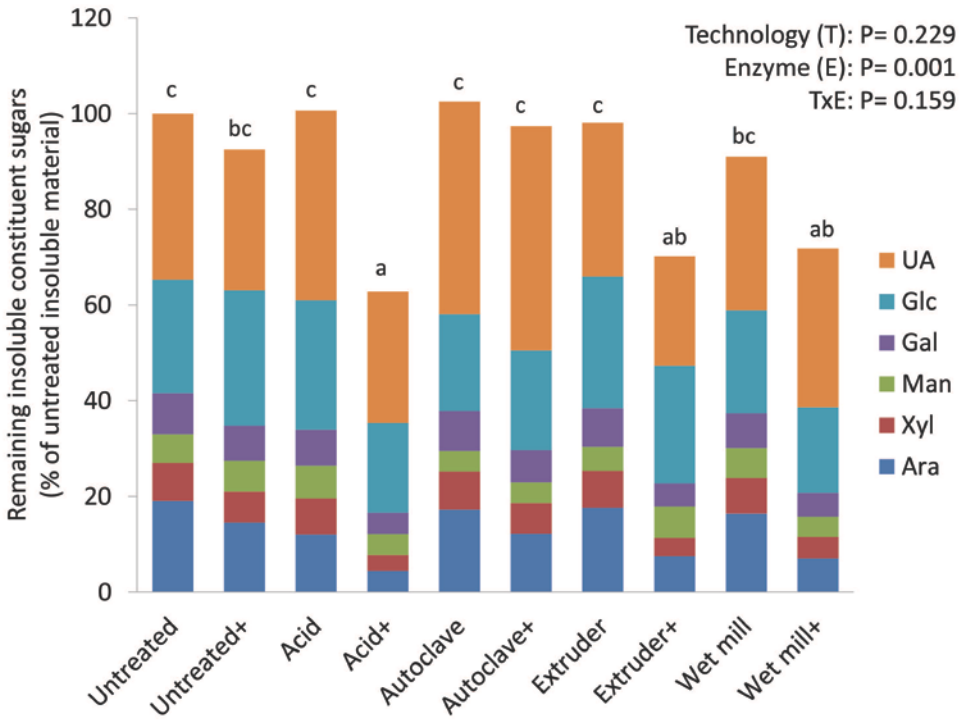

Figure 3.1. Constituent NSP sugars in insoluble fraction after *in vitro* incubation of (pre-treated) RSM. Ara = arabinose, Xyl = xylose, Man = mannose, Gal = galactose, Glc = glucose, UA = uronic acid, + = with the addition of commercial pectinolytic enzymes. Bars with the same letter on top represent total sugar levels that are not significantly different.

With regard to individual constituent sugars, large differences were seen between pre-treatments. An obvious decrease was determined for polymeric arabinosyl (shown in Figure 3.2) and xylosyl (not shown) after the mild acid pre-treatment. These five-carbon sugars are known to be more acid-labile than six carbon sugars (*29*).

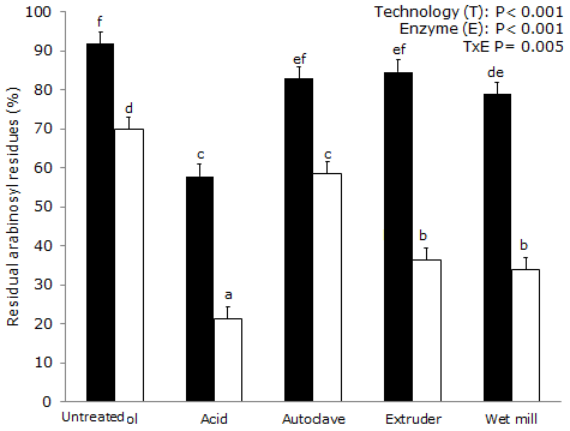

Figure 3.2. Residual arabinosyl residues (as percent of starting material) after *in vitro* incubation of (pre-treated) RSM either without (black bars) or with the addition of commercial pectinolytic enzymes. Bars with the same letter on top are not significantly different.

The studied pre-treatments, without commercial pectinolytic enzyme addition, hardly affected the contents of mannosyl, glucosyl, and uronyl residues (Figure 3.1). After commercial pectinolytic enzyme addition to untreated, wet-milled, extruded, and mild acid pre-treated RSM, fewer arabinosyl residues remained than without the addition of commercial pectinolytic enzymes (70, 34, 36, and 21% remaining after *in vitro* incubation, respectively). Apparently, arabinan in RSM is rather well enzyme degradable when preceded by a pre-treatment. After commercial pectinolytic enzyme addition to mild acid pre-treated RSM, also fewer uronyl residues remained than without commercial pectinolytic enzyme addition.

The WBC of the incubated residues is shown in Figure 3.3-A. For untreated RSM it was 9 g water/g material (or 5 g water/g starting material, Figure 3.3-B), whereas untreated RSM that has not been digested *in vitro* binds 2.4 g water/g material (Table 3.1). Expression in grams of water bound per gram of starting material takes into account that less DM remained after *in vitro* incubation. An increase in WBC during *in vitro* incubation means that the cell wall structure is affected in such a way that more water can be held per gram of material. Addition of commercial pectinolytic enzymes did not have an effect on the WBC of untreated RSM. After commercial pectinolytic enzyme addition to wet-milled, extruded, and mild acid pre-treated RSM, the WBC of the *in vitro* incubation residue

decreased to 5.6, 6.4, and 6.5 g water/g residue, respectively, compared to without commercial pectinolytic enzyme addition.

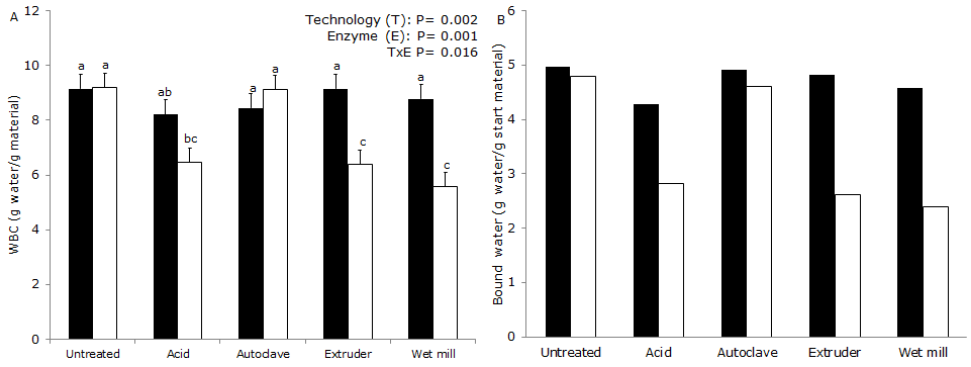

Figure 3.3. Water-binding capacity (g water/g dry matter) of starting material and residues from *in vitro* incubation of (pre-treated) RSM either without (black bars) or with (white bars) the addition of commercial pectinolytic enzymes: (A) WBC represented as g water/g residue from *in vitro* incubation; (B) WBC represented as g water/g starting material for *in vitro* incubation. Bars with the same letter on top are not significantly different.

When WBC was expressed as grams of water bound per gram of starting material, there was a decrease compared to the untreated RSM after *in vitro* incubation (2.4–2.8 and 4.8 g water/g starting material, respectively). As expected, cell wall material is degraded by the addition of commercial pectinolytic enzymes so extensive that the material can hold less water.

2.3.2 Viscosity and molecular weight distribution of soluble polysaccharides from in vitro *incubation*

The intestinal viscosity is partly determined by the soluble polysaccharides in the digesta and influences the passage rate *in vivo* (*28*). Therefore, the soluble fraction of the *in vitro* incubation was analyzed for its viscosity (Figure 3.4) together with the apparent molecular weight of the solubilized polysaccharides (HPSEC; Figure 3.5). It should be noted that viscosity continuously changes as digestion proceeds (*30*), and only the end point has been analyzed in this study. Nevertheless, the values indicate trends in viscosity changes provoked by the various treatments.

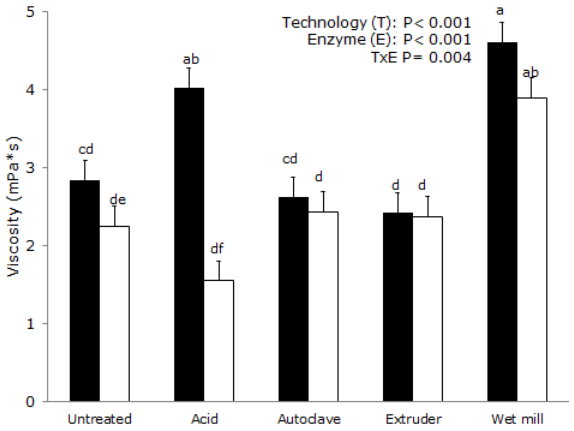

Figure 3.4. Viscosity (mPa·s) of solubles from *in vitro* incubation of (pre-treated) RSM either without (black bars) or with (white bars) the addition of commercial pectinolytic enzymes. Bars with the same letter on top are not significantly different.

The viscosity of the soluble fraction of the incubation of untreated RSM was 2.8 mPa·s. This value is in the same order of magnitude as the viscosity of *in vivo* digesta at the end of the small intestine for pigs fed a wheat-containing diet (*31*). Wet milling and mild acid pre-treatment increased viscosity to 4.6 and 4 mPa·s (Figure 3.4), respectively, even though similar amounts of sugars were solubilized (assuming no sugars were lost). In Figure 3.5, it can be seen that high molecular mass material (peak A; >400,000 Da) was solubilized during *in vitro* incubation of untreated RSM. From wet-milled RSM, the area under peak A increased 1.8 times, meaning that more of this molecular weight population was solubilized during wet-milling compared to untreated RSM. Simultaneously, viscosity increased 1.6 times by wet-milling compared to untreated RSM. With the addition of commercial pectinolytic enzymes to wet-milled RSM, the area under peak A still increased 1.3 times compared to wet-milled RSM to which no enzymes have been added. Viscosity decreased slightly by enzyme addition to wet milled RSM, although not significantly. Apparently, besides increasing solubilization of material under peak A, the pectinolytic enzymes added also broke down interactions between carbohydrates, thereby lowering the viscosity. After *in vitro* incubation, in the supernatant of mild acid pre-treated RSM, peak A is absent and peak B (around 306,000 Da) appeared (Figure 3.5).

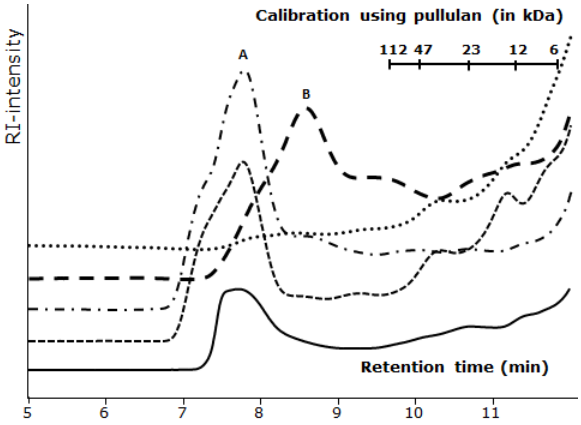

Figure 3.5. HPSEC elution pattern of supernatants after *in vitro* incubation of pre-treated RSM either without or with the addition of commercial pectinolytic enzymes compared to untreated RSM. From bottom to top: untreated (solid line), wet mill (square dotted line), wet mill with enzymes (dash-dotted line), mild acid (dashed line), and mild acid with enzymes (round dotted line).

After commercial pectinolytic enzyme addition, this peak B disappeared. Apparently, the mild acid pre-treatment made the NSP fraction better accessible for the commercial pectinolytic enzymes. Commercial pectinolytic enzyme addition to mild acid pre-treated sample decreased viscosity from 4.0 to 1.6 mPa·s, so peak B was responsible for the increase in viscosity when compared to untreated RSM (2.8 mPa·s). Peaks A (untreated) and B (mild acid pre-treated) were collected and analyzed for their glycosyl composition by methanolic HCl/TFA hydrolysis. Peak A was found to consist of 67 mol % glucosyl and 16 mol % xylosyl residues. Peak B was found to consist of 55 mol % glucosyl and 24 mol % xylosyl residues. Carbohydrates represented in peaks A and B were possibly xyloglucans, which are common in dicotyledonous plants (*32*). The xyloglucan in peak B has a higher xylose/glucose molar ratio than peak A (0.44 and 0.24, respectively). Arabinosyl, galactosyl, and mannosyl residues were found in equal ratios, which could be present as side chains on the xyloglucan.

3.3.2 In vitro *fermentation*

The washed residues from *in vitro* digestion were inoculated and fermented. The cumulative volume (DMCV) of gas produced is presented in Figure 3.6. In Figure 3.6-A the DMCV is expressed in milliliters of gas produced per gram residue of *in vitro* digestion, and in Figure 3.6-B the DMCV is expressed as milliliters of gas produced per gram of starting

material before *in vitro* incubation. Expression per gram starting material represents better the fermentability of the whole feed material than the amount of gas per gram residue, which is generally used in the literature (and shown in Figure 3.6-A). The effect of the *in vitro* digestion of (un)treated RSM was as such that most of the carbohydrates were solubilized and removed before fermentation. *In vivo* these soluble carbohydrates will also be available for fermentation by the microflora in the large intestine if they are not digested.

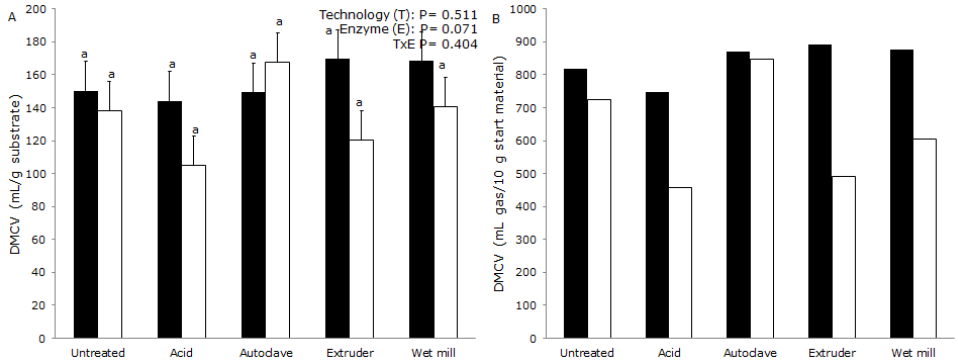

Figure 3.6. Cumulative gas produced during *in vitro* fermentation of (pre-treated) RSM either without (black bars) or with (white bars) the addition of commercial pectinolytic enzymes: (A) DMCV represented as milliliters of gas produced per gram residue from *in vitro* incubation; (B) DMCV represented as milliliters of gas produced per gram starting material for *in vitro* incubation. Bars with the same letter on top are not significantly different.

From samples with no commercial pectinolytic enzymes added, on average 84 mL of gas was produced per gram of starting substrate. No significant differences were seen between the different technological pre-treatments (P = 0.511). Although no significant difference was found, a trend is visible that addition of commercial pectinolytic enzymes to mild acid pre-treated, extruded, and wet-milled RSM decreases gas production compared to untreated RSM.

Commercial pectinolytic enzyme addition to autoclaved RSM did not show this trend in decreasing gas production. Overall, gas production from RSM was low when compared to other substrates, which were around 240 mL gas/g of organic matter after *in vitro* digestion of corn silage, rye grass, and Jerusalem artichoke (*13*). These raw materials are higher in neutral detergent fiber and lower in protein compared to RSM (*33*), which could explain the lower amount of gas produced. When less DM and less carbohydrate

remained in the residue after *in vitro* digestion, less gas seemed to be produced. This seems to be the trend for mild acid pre-treated, extruded, and wet-milled RSM to which commercial pectinolytic enzymes were added. Although differences in the sugar composition of the *in vitro* digestion residues exist (Figure 3.1), analysis of the fermentation residues showed very similar sugar compositions (data not shown).

In conclusion, from the different technological pre-treatments tested, mild acid pre-treatment only when used in combination with commercial pectinolytic enzymes showed to be the best in digesting RSM carbohydrates, because the least residual carbohydrate was found. Especially residual arabinosyl and uronyl contents decreased with mild acid pre-treatment and enzyme addition. This treatment also showed to be the best in decreasing the WBC of insoluble material and viscosity of soluble fraction. No significant differences between the treatments were seen in the fermentation of *in vitro* digestion resistant residues. Subsequent studies will be *in vivo* digestion trials using pre-treated RSM fed to chicken and pigs.

Acknowledgements

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Chapter 4

Effect of processing technologies and pectolytic enzymes on degradability of non-starch polysaccharides from rapeseed meal in broilers

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Submitted for publication.

Abstract

Rapeseed meal (RSM) contains a high level of non-starch polysaccharides (NSP) that are not well degraded in poultry and interfere with digestion of other nutrients as protein, starch, and fat. By altering physicochemical properties of NSP from RSM, processing and enzyme technologies might improve digestive utilization of RSM, enhancing its potential as a source of nutrients in poultry diets. The effects of wet-milling and extrusion in combination with pectolytic enzymes on the degradability of rapeseed meal in broilers were investigated in a 3 x 2 factorial arrangement. Wet-milling and extrusion did not affect total tract apparent digestibility of dry matter, crude protein, crude fat, and non-glucose polysaccharides (NGP). Addition of pectolytic enzymes did not affect total tract apparent digestibility of crude protein and crude fat, but improved degradability of NGP by 9 to 20 % units ($P < 0.001$), independent of prior technological processing of RSM. This coincided with an increase of the NGP concentration in the ceca with 4 to 7 g/g cobalt ($P < 0.001$). This indicated that NGP were solubilized such that they could enter the ceca and became available for fermentation. Wet-milling, known to reduce particle size, facilitated solubilization of polysaccharides from RSM, thereby, increasing the concentration of NGP found in the ceca with 4 g/g cobalt ($P = 0.008$). Without the help of additional pectolytic enzymes, those solubilized structures could, however, still not be degraded by the cecal microbiota. Feed intake, body weight gain, and gain:feed ratio were not affected. No interaction between processing technologies and enzyme addition was found. Apparently, the processing technologies studied were not facilitating accessibility of NSP to pectolytic enzymes added to the feed *in vivo*.

4.1 Introduction

Rapeseed meal (RSM), a by-product from rapeseed oil production, is a suitable protein source for poultry feed. However, its nutritional value is limited by the high level of non-starch polysaccharides (NSP) that typically constitute 20 to 40% of the meal (*1-4*). Non-starch polysaccharides can be partly fermented by the microbial community residing in the gastrointestinal tract, although in poultry, degradation is almost exclusively limited to the soluble fraction (*5*). In RSM only 8 to 15% of NSP is water-soluble, and NSP degradability values in chicken range from only 3 to 6% (*1, 6, 7*). In addition, NSP may affect digestion of other nutrients, both directly due to physical hindrance and indirectly due to physiological changes in the gut, such as increased digesta viscosity (*8*).

Processing technologies and cell wall degrading enzymes, such as pectinases, might be used to modify cell wall architecture and improve NSP degradability, as recently reviewed (*9*). Mechanical forces open the cell wall structure and reduce particle size, thereby increasing the surface area accessible for enzymes – i.e. both feed enzymes added to the diet and microbial enzymes present in the gastrointestinal tract. Hydrothermal treatment can break weak bonds between polysaccharides, but excessive heating may increase protein and free amino acid damage (*10*). In addition, thermal treatments often increase digesta viscosity (*9*), which can negatively affect nutrient digestion and absorption (*8*). In a previous *in vitro* study (*11*), effects of wet-milling – a technology that combines rigorous particle size reduction with moisturizing – and extrusion, combining hydrothermal treatment and shear, in combination with pectolytic enzymes were investigated. Although processing on its own did not improve NSP solubility *in vitro*, pectolytic enzymes were only effective when added to processed RSM, indicating that processing technologies are required to enable effective use of enzymes.

In the current study, the effects of particle size reduction (wet-milling) and hydrothermal treatment with shear (extrusion), in combination with pectolytic enzymes on the degradability of RSM in broilers, are investigated. It is hypothesized that: 1.) Processing technologies or pectolytic enzymes increase NSP degradability of RSM, where the extent of improvement will depend on the mode of action of the technology and 2.) The effectiveness of pectolytic enzymes to improve NSP degradability depends on the extent to which the cell wall structure is modified during processing.

4.2 Materials and methods

4.2.1 Experimental design

Effects of processing technologies and pectolytic enzymes on degradation of RSM were tested in a 3 x 2 factorial arrangement: three processing technologies (unprocessed, wet-milling, and extrusion-cooking), each with or without enzyme addition. The experiment was conducted at research farm 'De Haar' of Wageningen University. All experimental procedures were approved by the Animal Care and Use Committee (DEC) of Wageningen University. Enzyme use was approved by the Dutch Ministry of Agriculture, Nature, and Food Quality.

4.2.2 Materials and diets

Rapeseed meal (*Brassica napus*, Cargill N.V., Antwerp, Belgium; 2011) was obtained from a commercial feed mill (Agrifirm B.V., Utrecht, The Netherlands). Six diets, containing 35% unprocessed or processed RSM, and two basal diets (with and without pectolytic enzymes), were formulated (Table 4.1) to meet or exceed nutrient requirements of broilers (*12*). Basal diets were used to calculate RSM degradability using the difference method. Rapeseed meal was processed at Wageningen Feed Processing Centre (Wageningen University, Wageningen, The Netherlands). Diets were fed as pellets; product temperature during pelleting was 65 to 70°C.

4.2.3 Processing technologies

4.2.3.1 Wet-milling

Rapeseed meal was milled using a laboratory-scale refiner (Sprout-Waldron, Muncy, PA, USA) at a feed rate of 158 $kg \cdot h^{-1}$ using 480 liter water per h. Diameter of the discs was 30 cm with the distance between discs set at 0.07 mm and a rotation speed of 3000 rpm. Product temperature when leaving the machine was 32 ± 5.8°C.

Table 4.1. Composition of basal and rapeseed meal diets (%, as-fed basis, unless indicated otherwise)

	Basal diets	Rapeseed meal diets
Ingredient		
Rapeseed meal	-	35.0
Maize	46.2	30.0
Maize starch	24.1	15.8
Wheat gluten meal	7.7	5.0
Fishmeal	6.2	4.0
Soy protein isolate	3.9	2.5
Soy oil	6.9	4.5
Limestone	1.08	0.70
Mineral and vitamin premix [a]	0.50	0.50
Monocalcium phosphate	0.69	0.45
Potassium bicarbonate	1.06	0.30
Salt	-	0.10
Sodium bicarbonate	0.17	0.11
L-Lysine HCL	0.46	0.30
D- Methionine	0.28	0.18
L-Threonine	0.12	0.08
L- Valine	0.15	0.10
L-Tryptophan	0.06	0.04
L-Isoleucine	0.12	0.08
L. Arginine	0.18	0.12
Co-EDTA	0.10	0.10
Cr_2O_3	0.025	0.025
Calculated nutrient composition [b]		
ME, kcal/kg	3344	2627
Calcium	0.72	0.76
Available phosphorus	0.28	0.31
Sodium	1.7	1.6
Digestible lysine	0.99	1.16
Digestible methionine + cysteine	0.84	0.95
Digestible threonine	0.56	0.78

[a] Provided per kilogram of diet: Vitamin A (retinyl acetate), 12.000 IU; cholecalciferol, 0.6 mg; vitamin E (DL-α-tocopherol), 50 mg; vitamin B_2 (riboflavin), 7.5 mg; vitamin B_6 (pyridoxine-HCl), 3.5 mg; vitamin B_1 (thiamin), 2.0 mg; vitamin K (menadione), 1.5 mg; vitamin B_{12} (cyanocobalamin), 20 µg; choline chloride, 460 mg; anti-oxidant (oxytrap PXN), 125 mg; niacin, 35 mg; d-calcium pantothenate, 12 mg; biotin, 0.2 mg; folic acid, 1 mg; Mn, 85 mg, as MnO; Fe, 80 mg, as $FeSO_4$; Zn, 60 mg, as $ZnSO_4$; Cu, 12 mg, as $CuSO_4$; I, 0.8 mg, as KJ; Co, 0.4 mg, as $CoSO_4$; Se, 0.15 mg, as Na_2SeO_3. [b] According to the Dutch CVB (*12*).

4.2.3.2 Extrusion-cooking

Water was added to the unprocessed products to reach a DM content of 75 % (w/w) and samples were mixed using a paddle-mixer (Type F60; Halvor Forberg, Bygland, Norway). Within 60 minutes after mixing, samples were extruded using a co-rotating double screw extruder (M.P.F.50; Baker Perkins, Peterborough, UK) with a length/diameter ratio of 25. The screw configuration was as follows: four 1.5D feed screw elements, one 1D single lead element, three 1D feed screw elements, one 1D single lead element, two 1D feed screw elements, two 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, one 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, two 4D 90 degree forwarding block paddles, and two 1.5D single lead elements. A die with two orifices (6 mm) was used; no die face cutter was used. Feeding rate was 36 $kg \cdot h^{-1}$, screw speed was 250 rpm. Barrel temperatures in the ten segments of the extruder were set at 30, 50, 72, 82, 90, 105, 115, 120, 120, and 120 °C. Product temperature at the die was 110 ± 1.6 ºC, pressure at the die was 493 ± 7.1 psi.

Wet-milled and extruded RSM meal were vacuum dried (35-VDC-45, Hosokawa Micron, Doetinchem, The Netherlands) using a barrel temperature of 50 ºC and a vacuum set point of 1000 Pa.

4.2.4 Cell wall degrading enzymes

The enzyme treatment comprised a combination of the commercial enzyme mixtures Pectinex UltraSP-L (Novozymes, Bagsvaerd, Denmark) and Multifect Pectinase FE (DuPont Industrial Biosciences, Genencor division, Rochester, NY, USA), with mainly pectolytic and some hemicellulolytic activities. Enzymes were added to the unprocessed or processed RSM (25 mL/kg DM) and incubated for 1 h, before mixing in the diet.

4.2.5 Birds and experimental procedures

A total of 750 1-day-old chicks (initial body weight 40.7 ± 3.33 g; Ross 308, Aviagen Group, Newbridge, United Kingdom), obtained from a commercial hatchery (Broederij Lagerweij B.V., Lunteren, The Netherlands) were housed in 10 floor pens bedded with wood pellets (SOFTCELL, Agromed GmbH, Kremsmünster, Austria). All broilers were fed a starter diet with 20% (w/w) RSM, containing 21% (w/w) crude protein (CP) and providing 2743 kcal/kg metabolizable energy. At day 14, 576 broilers were allocated to one of the 8 diets (Tables

4.1 and 4.2) based on bodyweight, and housed in metabolism cages (65 x 75 cm). In total, the experiment comprised 48 cages, with 6 cages per treatment. Each cage housed 12 broilers from day 14 to day 25 and 11 broilers from day 26 to the end of the experiment. Average bodyweight of the broilers at day 14 was 349.7 ± 37.7 g. Feed was available *ad libitum* and broilers had free access to water during the experiment. Ambient temperature was maintained 33°C at day 1 and gradually decreased to 21°C at day 25. Photoperiod was 23L:1D during the first two days and 16L:8D from day 3 onwards. Broilers were spray-vaccinated against Newcastle disease at day 15.

After a 14 day pre-experimental period when all chicks received the starter diet, the experiment consisted of a 10 day adaptation to the experimental diets, followed by 4 days collection of excreta. At the end of the experiment (day 30, 31, or 32) animals were euthanized and contents from crop, ileum (last 50 cm before the ileo-cecal junction), and ceca were collected. Broilers were fasted from 6 to 3 h before euthanasia and subsequently allowed to feed from 3 h before euthanasia onwards. Feed intake per cage was recorded throughout the experiment. Broilers were weighed at the start of the experiment (day 1), adaptation period (day 14), excreta-collection period (day 25), and at the end of the experiment (day 30, 31, or 32). Excreta were collected quantitatively. Gastrointestinal contents and excreta were pooled per cage and immediately frozen (-20 °C). Contents from ileum and ceca, as well as excreta were freeze-dried. Crop contents were dried in a vacuum stove (4h, 80 °C).

4.2.6 Analytical methods

The water binding capacity (WBC) of diets and crop contents was analyzed in duplicate by soaking 1 gram of raw material or dried crop content in 25 mL of water for 24 h at room temperature. Samples were centrifuged at 3274 *g* for 20 min at room temperature and decanted. Water binding capacity was calculated as the weighed quantity of water retained per g of dry material.

Prior to chemical analyses, samples were milled in a mixer mill (MM 2000, Retsch GmbH, Haan, Germany) at amplitude of 80, during 1 min. All chemical analyses were performed in duplicate using standard laboratory methods (*13, 14*). Diets, gastrointestinal contents, and excreta were analyzed in duplicate for contents of DM (AOAC 930.15), ash (AOAC 942.05), cobalt (by atomic absorption spectrophotometry, using a SpectrAA 300 atomic absorption spectrophotometer, Varian B.V., Middelburg, The Netherlands), nitrogen (diets by Kjeldahl

method: ISO 5983. Gastrointestinal contents and excreta by Dumas method: AOAC 990.03; using a Thermo Quest NA 2100 Nitrogen and Protein Analyzer, Interscience B.V., Breda, The Netherlands), total starch (AOAC 996.11; using a commercial test kit, Megazyme international Ltd., Bray, Ireland), and total NSP measured as neutral sugars and uronic acids. Neutral sugar composition was analyzed by gas chromatography according to the method of Englyst and Cummings (*15*). After pre-treatment with 72 % (w/w) H_2SO_4 for 1 h at 30 °C, samples were hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. Constituent sugars were derivatized into alditol acetates and analyzed using a GC (Focus-GC, Thermo Scientific, Waltham, MA). Inositol was used as internal standard. Uronic acid content was analyzed according to the automated colorimetric m-hydroxydiphenyl assay (*16*), including tetraborate, using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration. Non-starch polysaccharide content was calculated as the sum of neutral sugars and uronic acids minus glucose from starch. Nitrogen content of diets was corrected for differences between analytical methods (Kjeldahl vs. Dumas method) using: $N_{Dumas} = N_{Kjedahl} \times 0.9885 + 0.0103$ (*17*). Crude protein content was calculated as Nitrogen content x 5.3 (*18*). Fecal nitrogen in the excreta was calculated as total nitrogen minus nitrogen in uric acid. Uric acid was analyzed enzymatic-colorimetric using a commercial test kit (10694, Human GmbH, Wiesbaden, Germany). Diets and excreta were analyzed for content of ether extract using Soxhlet apparatus and petroleum ether, after hydrochloric acid hydrolysis (AOAC 920.39). Diets were additionally analyzed for contents of neutral detergent fiber (NDF; AOAC 2002.04) and glucosinolates (ISO 9167-1), as well as phytate, total phosphorus, and phytate bound phosphorus (enzymatic-colorimetric; using a commercial test kit, K-PHYT 07/11, Megazyme international Ltd., Bray, Ireland).

4.2.7 Calculations and statistical analysis

Total tract apparent digestibility (TTAD) of RSM was calculated using the difference method (*19*). Small amounts of residual starch (2 to 3 g/100 g DM, data not shown) were present in excreta. Therefore, TTAD of non-glucose polysaccharides (NGP; NSP – glucose) was calculated. Total tract apparent digestibilities of starch, NSP, and NGP were calculated using the unprocessed control diet as the reference.

Data were analyzed by analysis of variance, with the GLM procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA), using processing technology, enzyme addition, and their

interaction as fixed effects. The effects of the blocking factor dissection day and its interaction with dietary treatment were tested, but found not to be significant and excluded from the model. Cage was the experimental unit. Model residuals were tested for homogeneity and normality, to verify model assumptions. Least square means (LSMeans) were compared using Tukey adjustments for multiple comparisons. Data are presented as LSMeans and pooled standard error of the mean (SEM) unless stated otherwise. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

4.3 Results and discussion

4.3.1 Bird performance

In two cages (basal-enzyme diet and unprocessed RSM diet), one or more broilers suffered from *E. Coli* infection, as confirmed by laboratory analyses of excreta (Dutch animal health service, Deventer, The Netherlands). These cages were excluded from statistical analyses. In one cage (extruded RSM diet), feed intake between day 14 and 25 was not recorded and this cage was excluded from performance analyses.

The analyzed chemical compositions of basal and RSM diets are presented in Table 4.2. Phytate and phytate bound phosphorus contents were in the range expected (*12, 20*) and differences between diets were small. The glucosinolate contents (calculated to be < 3 µmol/ g DM RSM) of the RSM used in the current study was low (*20*). Consequently, the effects of processing on glucosinolates and myrosinase activity were minor. The WBC varied between RSM diets (1.7 to 2.4 g/g DM), with extruded RSM diets showing the highest WBC. Apparently, the combination of heat, pressure, and shear opened the RSM cell wall matrix, confirming previous observations (*11*).

Table 4.2. Analyzed chemical composition and water binding capacity (WBC) of basal diets and RSM diets with (+) and without (-) addition of pectolytic enzymes (%, DM basis [a]).

	Basal diet		Unprocessed RSM		Wet-milled RSM		Extruded RSM	
Item	-	+	-	+	-	+	-	+
DM, % as fed	89.2	88.9	90.0	90.4	88	88.1	91.9	91.7
Crude protein	17.9	17.6	23.7	24.3	24.0	23.6	24.4	23.8
Starch	57.3	54.3	35.1	33.9	35.4	38.8	34.1	33.6
Crude fat	10.3	10.3	9.2	9.2	9.5	9.2	9.4	9.1
Ash	4.6	4.7	5.5	5.4	5.5	5.6	5.5	5.4
NDF [b]	5.7	5.6	13.7	13.7	13.3	13.5	13.7	14.5
NSP [c]	6.8	5.6	17.6	17.2	18.0	18.0	18.0	16.3
Molar composition of NSP [c]								
Rhamnose	0	0	1	1	1	1	1	1
Arabinose	24	24	22	21	21	22	21	22
Xylose	41	39	16	16	15	17	15	16
Mannose	6	7	3	3	3	3	4	3
Galactose	6	6	7	6	6	7	7	7
Glucose	11	9	30	29	31	29	30	29
Uronic acid	12	13	21	24	23	22	22	23
Phosphorus	0.63	0.53	0.57	0.53	0.65	0.65	0.59	0.55
Phytate bound	0.17	0.23	0.32	0.28	0.34	0.30	0.34	0.31
Glucosinolates (µmol/g DM)	0.7	1.0	1.2	1.4	0.9	0.9	1.4	1.2
WBC (g/g DM)	1.5	1.6	1.7	1.8	2.1	2.0	2.4	2.3

[a] Unless indicated otherwise. [b] NDF: Neutral detergent fiber. [c] NSP: Non-starch polysaccharides. [c] Mol %; presented as anhydrous sugar moieties.

Table 4.3. Growth performance of broilers fed diets containing unprocessed, wet-milled, or extruded RSM with (+) or without (-) addition of pectolytic enzymes [a].

Item	Unprocessed RSM diet		Wet-milled RSM diet		Extruded RSM diet		Pooled SEM	Model P-value[b]		
	-	+	-	+	-	+		Processing (P)	Enzyme (E)	PxE
n [c]	5	6	6	6	5	6				
ADG [d], g/bird	64.1	62.4	63.5	64.7	63.7	63.3	3.20	0.788	0.794	0.505
ADFI [e], g DM/bird	81.3	81.1	82.2	81.5	79.3	80.0	2.29	0.060	0.933	0.718
G:F [f], g:g	0.79	0.77	0.77	0.80	0.81	0.79	0.035	0.339	0.765	0.258
N retention (g/g bird per day) [g]	4.69	4.78	4.83	4.52	4.69	4.77	0.36	0.905	0.691	0.312
N retention (%) [h]	56.4	59.4	56.4	55.0	54.9	56.5	2.92	0.125	0.278	0.180

[a] Recorded from 14 to 25 days of age, except for N retention, which was recorded from 26 to 29 days of age. [b] Effect of processing technology (P), addition of pectolytic enzymes €, or their interaction (PxE). [c] Number of replicate cages of 12 broilers each. [d] ADG: Average daily gain. [e] ADFI: Average daily feed intake. [f] G:F: Gain to feed ratio. [g] Nitrogen retention (g/ bird per day). [h] Nitrogen retention relative to nitrogen intake (%).

Average daily gain and nitrogen retention did not differ between dietary treatments (Table 4.3). When corrected for dry matter content of the feed, also averaged daily feed intake (ADFI) and gain to feed ratio (G:F) did not differ between dietary treatments.

4.3.2 Nutrient digestion and physicochemical properties of digesta

Total tract apparent digestibility of crude protein and crude fat were affected by neither processing technologies nor by enzyme addition (Table 4.4, Figure 4.1). Total tract apparent digestibility of dry matter mainly followed differences in NGP degradability as described below.

Physicochemical properties of the diet and digesta, as particle size, WBC, and viscosity, may affect gastrointestinal retention time (*21-24*), thereby potentially affecting nutrient digestibility in the small intestine.

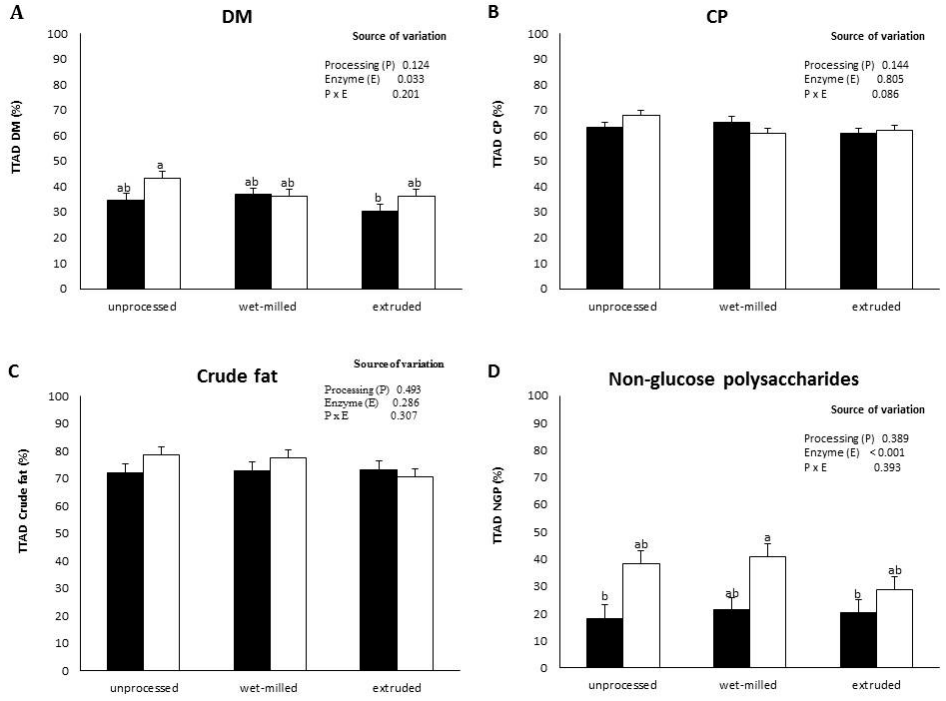

Figure 4.1. Total tract apparent digestibility (TTAD) of dry matter (DM; A),crude protein (CP; B), crude fat (C), and non-glucose polysaccharides (NGP; D) from unprocessed, wet-milled, or extruded rapeseed meal (RSM) with (white bars) and without (black bars) addition of pectolytic enzymes when fed to broilers. Error bars indicate SEM. Means lacking a common data label (a,b) differ ($P < 0.05$).

Table 4.4. Total tract apparent digestibility (TTAD) of dry matter and nutrients of diets containing unprocessed, wet-milled, or extruded RSM with (+) or without (-) addition of pectolytic enzymes when fed to broilers.

Item	Unprocessed RSM diet		Wet-milled RSM diet		Extruded RSM diet		Pooled SEM	Model P-value [1]		
	-	+	-	+	-	+		Processing (P)	Enzyme (E)	PxE
n [2]	5	6	6	6	6	6				
TTAD, %										
Dry matter	66.5 [ab]	70.1 [a]	67.3 [ab]	67.6 [ab]	65.1 [b]	67.6 [ab]	2.22	0.124	0.009	0.201
Crude protein	71.5	74.4	72.6	70.7	70.4	71.4	4.15	0.143	0.434	0.085
Crude fat	88.2	90.2	88.5	89.9	88.6	88.0	3.55	0.491	0.180	0.306
NSP [3]	19.8	35.8	19.8	36.8	22.5	28.1	8.56	0.743	<0.001	0.329
NGP [4]	24.2	37.9 [ab]	26.1 [bc]	39.6 [a]	25.5 [bc]	31.8 [abc]	7.27	0.389	<0.001	0.393

[1] Effect of processing technology (P), addition of pectolytic enzymes (E), or their interaction (PxE). [2] Number of replicate cages of 11 broilers each. [3] Non-starch polysaccharides. [4] Non-glucose polysaccharides. [a, b, c] Means within a row lacking a common superscript differ (P<0.05).

Addition of pectolytic enzymes decreased WBC of crop contents (Supplemental figure 4.1B), indicating that the feed matrix was affected to hold less water, possibly due to hydrolysis of RSM NSP. Dry matter content in the crop was affected by processing (Supplemental figure 4.1A), possibly reflecting differences in digesta viscosity resulting from processing, as found *in vitro* (*11*). Dry matter content of small intestinal digesta tended to show the same trend (P = 0.058; Appendix Figure 4.1C).

4.3.3 Non-glucose polysaccharide degradation

Enzyme addition increased TTAD of NGP from unprocessed and processed RSM (9 to 20 % units, *P* < 0.001; Figure 4.1D). This coincided with an increase (4 to 7 g/g cobalt, *P* < 0.001) in NGP concentration in the ceca (Figure 4.2), indicating that either less NGP that had entered the ceca were fermented or that a higher quantity of NGP was solubilized and actually entered the ceca. The positive correlation (r = 0.53, *P* = 0.001) between the NGP concentration of ceca contents and the TTAD of NGP supports the latter explanation, which matches well with our expectations and previous *in vitro* results (*11*).

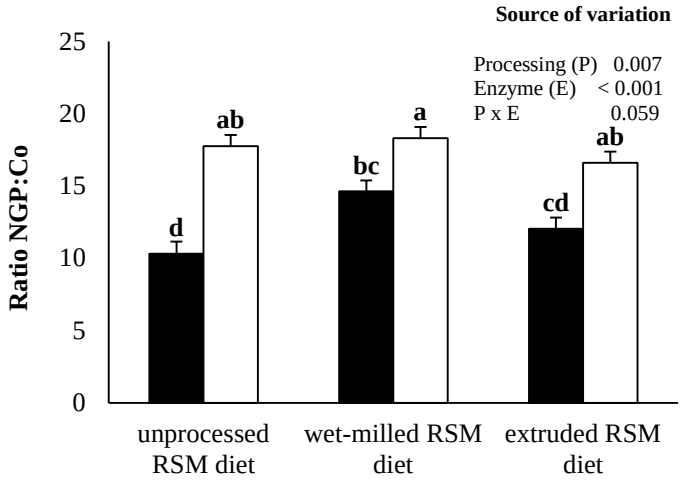

Figure 4.2. Non-glucose polysaccharide content expressed relative to Co-EDTA marker in the ceca contents from broilers fed diets containing unprocessed, wet-milled, or extruded rapeseed meal (RSM) with (white bars) and without (black bars) addition of pectolytic enzymes. Error bars indicate SD of total non-glucose polysaccharides.

The undegraded constituent monosaccharides are presented in Figure 4.3. The figure indicates that mainly arabinosyl residues (TTAD increased by 15 to 19 % units, $P < 0.001$, data not shown), and to a lesser extent uronyl residues (6 to 13 % units, $P = 0.006$, data not shown) were affected. Detailed analysis of residual carbohydrate structures in excreta, revealed that the pectolytic enzymes added to the diets mainly affect the degradation of branched water-soluble arabinan (*25*).

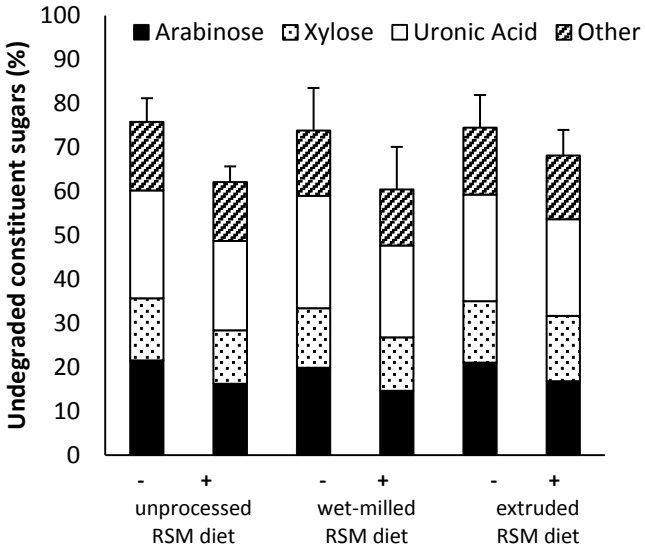

Figure 4.3. Undegraded constituent sugars in excreta (% of non-glucose polysaccharides consumed) from broilers fed diets containing unprocessed, wet-milled, or extruded rapeseed meal, with (+) and without (-) addition of pectolytic enzymes.

Despite improved NGP degradability, no differences in performance between birds fed RSM diets with or without pectolytic enzymes were observed. The expected improvement in growth as a result of increased NGP degradability in the ranges found is small (calculated to be < 1 g/d, assuming an energy requirement of 10 to 17 kcal ME/g (*26, 27*).
Total tract apparent digestibility of NGP was not affected by processing technologies (Table 4.4, Figure 4.1D). Regardless, wet-milling increased NGP concentration of ceca contents (with 4 g/g cobalt, $P = 0.008$), compared with unprocessed RSM (Figure 4.2), indicating that more NGP have been solubilized and, therefore, could enter the ceca. These findings fit well with what one would expect based on the smaller particle size of

wet-milled RSM compared with unprocessed RSM and the higher NGP solubilization of wet-milled RSM found *in vitro* (*11*). A similar, but not significant, response was observed for extruded RSM diets. Nevertheless, processing did not improve TTAD of NGP, indicating that although polysaccharides were solubilized as a result of particle size reduction (*11*), structures could still not be degraded by the cecal microbiota.

No interaction between processing technologies and enzyme addition on NGP degradation was found. *In vitro*, processing of RSM was required to enable enzymes to effectively improve NSP solubilization (*11*), suggesting that the cell wall architecture is limiting the enzyme accessibility in unprocessed RSM. In the current study enzymes were also effective in unprocessed RSM diets, indicating that, *in vivo*, the processing technologies studied did not facilitate accessibility of NSP to pectolytic enzymes added to the diet. Possibly, physico-chemical modifications that occur during the digestion process in the bird, sufficiently opened the cell wall architecture to enable effective accessibility of NSP to pectolytic enzymes. Alternatively, NSP might have been better accessible to pectolytic enzymes in the current RSM batch, which had a relatively high content of soluble NSP (3.7 g/ 100g DM) compared with the RSM batch used previously (2.6 g/ 100 g DM; (*28*)).

Total tract apparent digestibility of NSP in the current study was higher than values reported previously for broilers and laying hens (~ 11 to 17 % units, (*1, 6, 7, 29*)). The high variation in NSP content as well as in composition between various batches of RSM (*2, 6*) can have considerable effects for its degradation in the bird (*6*). In agreement with what one would expect based on ceca physiology (*21, 30, 31*), fermentative degradation of NSP in poultry is almost exclusively limited to the soluble fraction (*5*). Therefore, the relative high content of soluble NSP in the RSM batch studied (3.7 compared with ~ 2 g/100 g DM found in above mentioned studies) may, in part, be responsible for the high extent of NSP degradation found. In addition, the long adaptation period to RSM in the current study, may contribute to these findings.

In conclusion, the pectolytic enzymes used, effectively modified the cell wall structure of RSM, resulting in increased NGP degradability by 9 to 20 % units ($P < 0.001$), independent of prior technological processing of RSM. This coincided with an increase in NGP concentration in the ceca of 4 to 7 g/g cobalt ($P < 0.001$), indicating that more NGP were solubilized such that they could enter the ceca and became available for fermentation.

Reduced particle size of RSM by wet-milling facilitated solubilization of polysaccharides from RSM, thereby increasing the concentration of NGP found in the ceca by 4 g/g cobalt (*P*= 0.008). Without help of additional pectolytic enzymes, those solubilized structures could, however, still not be degraded by the cecal microbiota. Feed intake, body weight gain, and gain:feed ratio were not affected. No interaction between processing technologies and enzyme addition was found. Apparently, the processing technologies studied did not facilitate accessibility of NSP to pectolytic enzymes added to the feed *in vivo*, opposed to results found *in vitro*.

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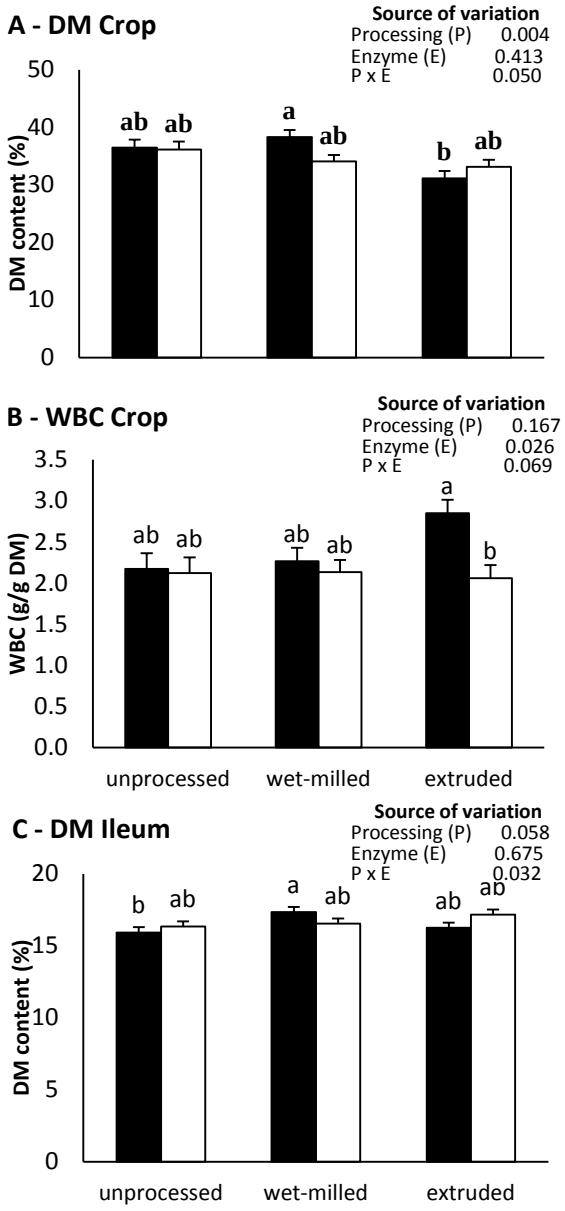
Supplemental data

Supplemental table 4.1. Non-glucose polysaccharide content (%w/w DM) in digesta in ileum and ceca and feces from broilers fed unprocessed, wet-milled, or extruded RSM with (+) or without (-) the addition of pectolytic enzymes.

	Unprocessed RSM		Wet-milled RSM		Extruded RSM	
	-	+	-	+	-	+
Ileum	14±5.0	19±1.9	18±3.8	18±1.8	20±1.3	18±2.7
Cecum	4±0.4	6±0.7	5±0.5	6±0.5	4±0.4	6±0.5
Feces	27±2.4	25±0.5	27±2.7	22±2.3	26±2.0	25±2.1



Supplemental figure 4.1. Dry matter (DM (A) and water binding capacity (WBC, B) of crop contents and DM content of ileum contents (C) from broilers fed diets containing unprocessed, wet-milled, or extruded RSM, with (white bars) or without (black bars) addition of pectolytic enzymes. Error bars indicate SEM. Means lacking a common data label (a, b) differ (P< 0.05).

Chapter 5

Carbohydrate structures resistant to fermentation in broilers fed (processed) rapeseed (*Brassica napus*) meal

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Abstract

Unprocessed and acid-extruded rapeseed meal (RSM) was fed to broiler chickens, with and without addition of commercial pectolytic enzymes. Unfermented non-starch polysaccharide (NSP) structures from RSM were studied in the excreta in detail. Mainly XXXG-type xyloglucan, (glucurono-)xylan, (branched) arabinan, and cellulose remained in the excreta. Addition of pectolytic enzymes decreased branchiness of the water-soluble arabinan, which most likely contributed to the significant increase in NSP-fermentability from 24 to 38% observed. Acid-extrusion was unable to improve NSP-digestibility significantly. Even, the proportion of unextractable carbohydrates increased in excreta from broilers fed acid-extruded RSM. Probably, acid-extrusion resulted in a less accessible NSP-matrix, also decreasing the accessibility for pectolytic enzymes added in the diet. During alkaline extraction of the excreta, 39-52% (w/w) of the insoluble carbohydrates was released as glucosyl- and uronyl-rich carbohydrates, probably originally present via ester-linkages or hydrogen-bonding within the cellulose-lignin network. These linkages are expected to hinder complete NSP-fermentation.

5.1 Introduction

Rapeseed meal (RSM) is a by-product from the production of rapeseed oil. After pressing and solvent-extraction of oil, the meal is dried by solvent-toasting and used as animal feed for its high protein content (around 38% w/w (*1*)). Apart from protein, RSM is rich in non-starch polysaccharides (NSP; 16-22% w/w (*3*)), which, after fermentation, potentially contribute to the energy supply. The NSP present in RSM are comprised of 1) pectic polysaccharides, e.g. homogalacturonan, rhamnogalacturonan, arabinan and arabinogalactan, 2) hemicelluloses, e.g. xyloglucan, glucuronoxylan and galactomannan, and 3) cellulose (*4, 5*).

In the small intestine of the chicken, enzymatic digestion of starch, small carbohydrates, proteins, lipids, and absorption of their degradation products takes place. Chickens lack the necessary digestive enzymes that can degrade NSP. Still, after the small intestine, the soluble fraction (and small particles) enters the caeca, where water is absorbed and water-solubles can be fermented until secretion (*6*). Also, reverse peristalsis in the colon refluxes the soluble fraction of digesta to the cecum to continue its fermentation (*7*).

In general, NSP-fermentability in broilers is low, which leaves part of the energy available in the feed unused. Fermentability of NSP from RSM is around 14%, while from soy bean meal around 20% of the NSP can be fermented by broilers (*8*). In addition, the presence of NSP in the feed reduces digestion of other nutrients due to their physicochemical properties, e.g. starch and protein digestion were reduced when isolated soluble NSP from wheat and rye were added to poultry diets (*9*).

An option to increase the energy value of RSM and to decrease the negative effect of NSP on other nutrients, is to enhance the degradability of these polysaccharides by processing of the RSM before inclusion into the diet. Hereto, different techniques like fine milling, extrusion, acid treatment, and enzyme addition have been tested *in vitro* (*10*). From that study, it was concluded that acid-treatment with addition of commercial pectolytic enzymes improves solubilization of NSP from RSM significantly. It is hypothesized in this *in vitro* study that the RSM cell wall structure is only accessible for enzymes added in the feed after technological treatment, as was also indicated by the interaction between technological treatment and enzyme addition found *in vitro* (*10*). As a follow-up experiment, diets containing this acid-treated RSM with and without the addition of commercial pectolytic enzymes were fed to broilers. The aim of the current paper is to identify which NSP-structures were recalcitrant to fermentation. Therefore, remaining

carbohydrate structures in the excreta of these broilers were analyzed in depth. This will assist in optimization of processing technologies.

5.2 Materials and methods

Chemicals were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) or VWR International (Radnor, PA, USA), unless stated differently.

5.2.1 In vivo trial: Animals and diets

In a 2x2 factorial arrangement, the effect of processing of RSM (unprocessed versus acid-extrusion) and enzyme addition (yes or no) was studied on the fate of the NSP fraction from RSM. The experimental procedures were approved by and conformed to the requirements of the Animal Care and Use Committee of Wageningen University, Wageningen, The Netherlands.

5.2.1.1 Animals and housing

Female one-day old broiler chickens (288 birds; Ross 308, Aviagen, Newbridge, United Kingdom; Hatchery Lagerweij, Lunteren, The Netherlands) were kept in floor pens for 14 days on wood pellets and then kept in metabolism cages (area: 65x75 cm) with 12 birds per cage for 7 days. At day 25, the number of birds per cage was standardized to 11. Ambient temperature was gradually decreased from 33°C at day 0 to 21°C at day 25. Relative humidity was maintained at a minimum level of 60% during the first three days and between 40 and 70% from day 3 onwards. Lighting was 23L:1D at day 0 and 1 (to assure a quick water and feed intake), and 16L:8D from days 2-30, according to European and Dutch regulations. All birds received New Castle disease-vaccination at 15 days of age.

5.2.1.2 Experimental diets

The ingredient composition of the experimental diets is presented in Table 5.1. Broilers had ad libitum access to diets in a pelleted form. Rapeseed meal (Cargill, Antwerp, Belgium, 2011, supplied by Agrifirm, Apeldoorn, The Netherlands) was included in the diet at a level of 35% (w/w). Besides RSM, also maize was included in the diet at a level of 30%, which contributes to the NSP content. The first diet contained unprocessed RSM

Table 5.1. Ingredient composition of the diets containing RSM (g/kg as fed) and analyzed contents of starch, protein, fat, and NSP (%w/w) and molar composition of NSP (mol%). Unpr. RSM: unprocessed RSM, Acid RSM: acid-extruded RSM, +: including addition of pectolytic enzymes.

	Diet Unpr. RSM	Diet Unpr. RSM+	Diet Acid RSM	Diet Acid RSM+
Unprocessed rapeseed meal	350	350		
Acid-extruded rapeseed meal			350	350
Maize	300	300	300	300
Maize starch	158.15	158.15	158.15	158.15
Wheat gluten meal	50	50	50	50
Soy oil	45	45	45	45
Fishmeal	40	40	40	40
Soy protein isolate	25	25	25	25
Limestone	7	7	7	7
Mineral and vitamin premix [a]	5	5	5	5
Monocalcium phosphate	4.5	4.5	4.5	4.5
Potassium bicarbonate	3	3	3	3
Sodium bicarbonate	1.1	1.1	1.1	1.1
Salt	1	1	1	1
L-lysine HCl	3	3	3	3
D-methionine	1.8	1.8	1.8	1.8
L-valine	1	1	1	1
L-threonine	0.8	0.8	0.8	0.8
L-isoleucine	0.8	0.8	0.8	0.8
L-tryptophan	0.4	0.4	0.4	0.4
L-arginin	1.2	1.2	1.2	1.2
Co-EDTA	1	1	1	1
Chromium oxide	0.25	0.25	0.25	0.25
Pectolytic enzymes [b]		8.75		8.75
Analyzed composition (%w/w dry matter)				
Starch	35	34	35	34
Protein	24	24	24	24
Fat	9	9	9	9
NSP [c]	17	17	16	11
Molar composition of NSP (mol%) [d]				
Rha	1	1	1	1
Ara	22	21	19	19
Xyl	16	16	14	12
Man	3	3	3	3
Gal	7	6	6	6
Glc	30	29	34	39
UA	21	24	23	20

[a] Provided per kilogram of diet: Vitamin A (retinyl acetate), 12.000 IU; cholecalciferol, 0.6 mg; vitamin E (DL-α-tocopherol), 50 mg; vitamin B_2 (riboflavin), 7.5 mg; vitamin B_6 (pyridoxine-HCl), 3.5 mg; vitamin B_1 (thiamin), 2.0 mg; vitamin K (menadione), 1.5 mg; vitamin B_{12} (cyanocobalamin), 20 µg; choline chloride, 460 mg; anti-oxidant (oxytrap PXN), 125 mg; niacin, 35 mg; d-calcium pantothenate, 12 mg; biotin, 0.2 mg; folic acid, 1 mg; Mn, 85 mg, as MnO; Fe, 80 mg, as $FeSO_4$; Zn, 60 mg, as $ZnSO_4$; Cu, 12 mg, as $CuSO_4$; I, 0.8 mg, as KJ; Co, 0.4 mg, as $CoSO_4$; Se, 0.15 mg, as Na_2SeO_3. [b] Enzymes were added as liquid (mL/kg feed). [c] NSP as extracted from the feed. [d] Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnose, Ara= arabinose, Xyl= xylose, Man= mannose, Gal= galactose, Glc= glucose, UA= uronic acid.

(Unprocessed RSM). The second diet contained unprocessed RSM with addition of commercial pectolytic enzymes (Unprocessed RSM+), the third diet contained acid-extruded RSM (Acid-extruded RSM), and the fourth diet contained acid-extruded RSM with addition of pectolytic enzymes (Acid-extruded RSM+). Prior to extrusion, RSM, water, and maleic acid were mixed using a paddle-mixer (Type F60, Halvor Forberg, Bygland, Norway) to a final contents of 19.8% (w/w) and 1.4% (w/w), respectively.

Within one hour, acid-extrusion of this mixture was performed with a double screw extruder (Baker-Perkins, Peterborough, UK) at 120˚C (product temperature 110.8±1.7˚C), 250 rpm screw speed, feeding rate 36 kg/h, and die size 6 mm. Commercial enzyme mixtures Pectinex UltraSP (Novozymes, Bagsvaerd, Denmark) and Multifect Pectinase FE (Genencor, Rochester, NY, USA) were added (8.75 mL per kilogram dry feed for each enzyme mixture) to the unprocessed RSM+ and acid-extruded RSM+ diets.

During days 26-29, excreta were collected quantitatively per cage to calculate digestibility parameters. Digestibility of dry matter, fat, and protein, and fermentability of NSP and constituent monosaccharides was calculated using the unprocessed RSM-diet as the reference. In two cages (unprocessed RSM and acid-extruded RSM+), one or more broilers suffered from *E. coli* infection, as confirmed by laboratory analyses of the Dutch animal health service (GD, Deventer, The Netherlands). Hence, these cages were excluded from analyses. To enable in-depth analysis of undigested carbohydrate structures, excreta samples (containing feces plus urine) of five or six cages were later pooled per treatment.

5.2.2 Extraction of water- and alkali-solubles from excreta

Unprocessed and acid-extruded RSM, and corresponding excreta were freeze-dried and milled using a ball mill (Retsch MM2000, Haan, Germany). The milled samples (2.5g) were extracted three times with 45mL demineralized water during 1 hour at 40°C. After each extraction, the solubilized material was separated from the insoluble residue by filtration over Whatman 595 ½ filters (Whatman, Kent, UK). Water-soluble fractions were combined, freeze-dried, and denoted as water-soluble solids (WSS). Water unextractable solids (WUS; ~0.75g) obtained were further extracted twice using 45mL of 6M NaOH containing 20mM $NaBH_4$ at 4°C. After each extraction, material was centrifuged (48,400*g*, 20 min, 4°C) and the supernatant was further separated from the insoluble residue by filtration on G2-glass filters. Alkali-soluble fractions were combined, neutralized using 6M HCl, dialyzed against demineralized water at 4˚C (Visking dialysis tubes, molecular mass

cut-off 12-14kDa, pore diameter ca. 25Å, Serva, Heidelberg, Germany), freeze-dried, and denoted as alkali-soluble solids (ASS). The final residue was neutralized using 6M HCl, washed with water, freeze-dried and denoted as residue (RES).

5.2.3 Enzymatic fingerprinting

WSS-, ASS- and RES-fractions from excreta (5 mg/mL) were incubated with pure, well-characterized enzymes. The choice for specific enzymes to demonstrate the presence of specific polysaccharides was based on the carbohydrate composition of these fractions. The enzymes used were polygalacturonase, rhamnogalacturonan hydrolase, β-galactosidase, endo-galactanase, endo-arabinanase, exo-arabinanase, a xyloglucan specific endo-glucanase, and endo-xylanase I, as described previously (*4*). Pure enzymes were dosed at 0.1 µg enzyme-protein per mg substrate. Besides these pure enzymes, a commercial cellulase preparation (CellicCTec, Novozymes) was used and dosed at 20 µg enzyme-protein per mg substrate.

The incubations were performed in 10 mM NaOAc buffer (pH 5) at 40˚C, rotating 'head-over-tail' for 24 hours. All enzymes were inactivated by heating at 100˚C for 10 minutes. Digests were analyzed by HPSEC, HPAEC, and MALDI-TOF MS.

5.2.4 Analytical methods

Dry matter content was determined in duplicate by drying overnight in an oven (WTC Binder, Tüttlingen, Germany) at 103°C.

Protein content (Nx5.3 (*11*)) was determined in duplicate by the Dumas method (*12*) on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Interscience, Troy, NY, USA). Samples (~10 mg) were weighed into a cup and directly analyzed. D-methionine was used for calibration.

Uric acid was analyzed enzymatic-colorimetric using a test kit (10694, Human GmbH, Wiesbaden, Germany). Nitrogen in the excreta was corrected for nitrogen from uric acid.

Total starch content was determined enzymatically using the total starch assay procedure K-TSTA 04/2009 (Megazyme, Bray, Ireland).

Fat content was determined by extraction with petroleum ether using the Soxhlet apparatus after hydrochloric acid hydrolysis (AOAC 920.39).

Non-starch polysaccharide extraction from the feed was performed as described elsewhere (*13*). In short, the starch present was gelatinized and enzymatically degraded, after which NSP could be precipitated using acidified ethanol.

Neutral carbohydrate content and composition was determined in duplicate according to Englyst and Cummings (*14*). After a pretreatment with 72% (w/w) H_2SO_4 for 1h at 30°C, the samples were hydrolyzed in 1M H_2SO_4 at 100°C for 3h. Afterwards, the monosaccharides released were derivatized into alditol acetates and analyzed using a GC (Focus-GC, Thermo-Fisher Scientific, Waltham, MA, USA). Inositol was used as internal standard.

Uronic acid content was determined in duplicate according to the automated colorimetric m-hydroxydiphenyl (Thermo Fisher Scientific) assay (*15*), including tetraborate, using an auto-analyzer (Skalar Analytical, Breda, The Netherlands). Galacturonic acid was used for calibration.

High Performance Size Exclusion Chromatography (HPSEC) was performed on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a set of four TSK-Gel superAW columns (Tosoh Bioscience, Tokyo, Japan) in series: guard column (6mm ID x 40mm) and separation columns 4000, 3000, and 2500 (6mm ID x 150mm). Samples (10µL) were eluted with aqueous 0.2M sodium nitrate at 55˚C at a flow rate of 0.6mL/min, followed by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan). Calibration was performed using pullulan standards of 180, 738 Da and 6, 12, 23, 47, and 112kDa. Enzyme digests were analyzed without prior dilution.

High Performance Anion Exchange Chromatography (HPAEC) was performed on an ICS-5000 System (Dionex) equipped with a CarboPac PA 1 column (2x250 mm) with pulsed amperiometric detection. Elution was performed at a flow rate of 0.3 mL/min and a temperature of 40˚C. The elution conditions used for quantification of fructose, saccharose, raffinose, and stachyose in WSS were: 0-5 minutes isocratic 0.1M NaOH (prepared with 50% (w/v) NaOH solution, Boom BV, Meppel, The Netherlands), 5-15 minutes linear to 0.1M NaOAc in 0.1M NaOH, 15-25 minutes linear to 0.3M NaOAc in 0.1M NaOH, isocratic for 5 minutes at 1M NaOAc in 0.1M NaOH, followed by 20 minutes isocratic on 0.1M NaOH. WSS-fractions (~5 mg/mL) were ten times diluted before analysis. Other elution conditions were used for the analysis of the enzyme digests: 0-45 minutes linear from 0.1M NaOH to 0.7M NaOAc in 0.1M NaOH, isocratic for 5 minutes on 1M

NaOAc in 0.1M NaOH, followed by 15 minutes isocratic on 0.1M NaOH. Enzyme digests were ten times diluted before analysis.

Matrix Assisted Laser-induced Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) was performed using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser of 337 nm and operated in the positive mode. After a delayed extraction time of 70 ns, the ions were accelerated to a kinetic energy of 25 kV and detected using a FlashDetector. The data were collected from averaging 200 laser shots, with the lowest laser energy needed to obtain sufficient spectra. External calibration was performed using maltodextrins (Paselli MD-20, AVEBE, Veendam, The Netherlands). Samples were desalted prior to analysis using AC 50W-X8 Resin (BioRad Laboratories, Hercules, CA, USA). 1µL desalted sample was mixed with 1µL matrix solution of 10 mg/mL 2,5-dihydroxy-benzoic acid (Bruker Daltonics) in 50% (v/v) acetonitrile and dried under a stream of air.

Glycosidic linkage type analysis was performed as described elsewhere (*16*). In short, polysaccharides were methylated, followed by hydrolysis with 2 M TFA for 1 hour at 121°C and permethylated monomers were converted into their alditol acetates. The treatment with methyliodide was performed twice. The partially methylated alditol acetates were identified by GC-MS using a Trace GC coupled to a DSQ-II (both Thermo Scientific) equipped with a Restek RTX-35MS column (Restek, Bellefonte, PA, USA). A temperature gradient was applied from 120°C to 250°C in 52 minutes, proceeded with a hold time of 5 minutes at 250°C. MS detection of masses 50-450 *m/z* was performed.

5.2.5 Statistical analysis

In a 2x2 factorial arrangement, the effects of acid-extrusion (T) and commercial pectolytic enzyme addition (E) were studied. The results from the *in vivo* experiment were statistically analyzed using the General Linear Models procedure with a SAS program (SAS Institute, version 9.2). The model used to describe the data was

$$Y_{ij} = \mu + T_i + E_i + T_i \times E_j + \varepsilon_{ij}$$

Where Y_{ij} is the response variable, μ is the overall mean, T_i the treatment (i= unprocessed, acid-extrusion), E_j the addition of commercial pectolytic enzymes (j= yes or no), $T_i \times E_j$ the interaction between treatment and enzyme addition, and ε_{ij} the error term. Residuals were tested for normality. For the excreta from broilers fed unprocessed RSM: n= 5, unprocessed RSM+: n=6, acid-extruded RSM: n=6, and acid-extruded RSM+: n=5.

5.3 Results and discussion

5.3.1 Characteristics of unprocessed and acid-extruded RSM

The RSM (Table 5.2; Unprocessed) contained 35% (w/w) proteins and 31% (w/w) carbohydrates, including 26% (w/w) NSP. Other compounds in RSM are expected to be lignin, lipids, and ash (*1*). RSM carbohydrates mainly contained glucosyl (40 mol%), arabinosyl (19 mol%), and uronyl (18 mol%) residues. As expected, the content and composition of RSM was not affected by acid-extrusion (Table 5.2). Nevertheless, the NSP-content as analyzed in the diet containing acid-extruded RSM+ was slightly lower (Table 5.1), which was a result of partly degradation by acid-extrusion and the pectolytic enzymes added, which were not analyzed as NSP in the feed, because these soluble carbohydrates are rinsed away in with starch in the analytical procedure for quantification of NSP from the feed. However, these soluble (partly degraded) NSP are present in the diets and still available for the animals.

Table 5.2. Composition of unprocessed and acid-extruded rapeseed meal (g/100g dry matter).

	Unpr. RSM [a]	Acid RSM [a]
Protein	35	34
Carbohydrate	31	30
Of which water-soluble saccharides (DP≤4) [b]	*17*	*15*
Of which water-soluble saccharides (DP>4) [b]	*12*	*12*
Of which water unextractable [b]	*71*	*73*
Others (lignin, ash, fat) [c]	34	36
Molar composition of carbohydrates [d]		
Rha	1	1
Ara	19	18
Xyl	8	8
Man	3	3
Gal	11	11
Glc	40	40
UA	18	19

[a] Unpr. RSM: unprocessed RSM; Acid RSM: acid-extruded RSM. [b] Percentage of water-soluble fructose, saccharose, raffinose, and stachyose (DP≤4), other water-soluble saccharides (DP>4), and water unextractable carbohydrates from total carbohydrates. [c] Calculated as dry matter minus protein and minus carbohydrates. [d] Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnose, Ara= arabinose, Xyl= xylose, Man= mannose, Gal= galactose, Glc= glucose, UA= uronic acid.

The molar carbohydrate composition of RSM (Table 5.2) indicated the presence of cellulose, xyloglucan, arabinan, and to a lesser extent homogalacturonan and arabinogalactan, as described before for *B. napus* meal (*4*). So, the commercial pectolytic enzymes added to the dry RSM are expected to work on the NSP-structures in RSM within the broiler's digestive tract. Even though the molar constituent monosaccharide composition was similar, in the RSM batch used in this study, the total carbohydrate content was lower compared to the RSM batch analyzed previously (*4*), where 36% (w/w) of carbohydrates was found. In the batch used for the *in vitro* study even 47% (w/w) of carbohydrates were found, with the surplus mostly caused by extra uronyl residues (*10*). The carbohydrate content in RSM is variable and can differ between cultivars (*17*) and can be affected by treatment during oil extraction (*18*). The NSP-content of 26% (w/w) found in this study is more in line with other values reported (16-22% (w/w) (*3*)) for industrially obtained RSM. In the diets, next to NSP from RSM, also NSP originating from maize was present (Table 5.1), which is estimated to be 15-20% (w/w) of the total NSP in the diet. Maize contains 6% (w/w) NSP, besides 62% (w/w) starch, 11% (w/w) protein, and some lignin. The molar composition of maize NSP has been reported as 33 mol% glucosyl, 30 mol% xylosyl, and 23 mol% arabinosyl residues, being present as mixed-linked β-glucans, glucuronoxylan, and cellulose (*19*).

5.3.2 In vivo fermentation of (enzyme-added) unprocessed and acid-extruded RSM

The gain to feed ratio of broilers fed unprocessed RSM was 0.79 kg growth/kg feed between day 14 and 25 of the trial. This was not affected by acid-extrusion (P=0.560), not affected by the addition of pectolytic enzymes (P=0.186), nor by a combination of acid-extrusion and enzyme addition (P=0.893).

Table 5.3. Total tract digestibility (%) of dry matter (DM), fat, and protein, and fermentability (%) of non-glucose polysaccharides (NGP), arabinosyl (Ara), xylosyl (Xyl), glucosyl (Glc), and uronyl (UA) residues from diets containing unprocessed or acid-extruded rapeseed meal with and without the addition of commercial pectolytic enzymes.

	Total tract digestibility and fermentability (%)							
Diet	DM	Fat	Protein	NGP	NSP	Ara	Xyl	UA
Unpr. RSM [a]	67	88	72	24	20	22	31	31
Unpr. RSM + [b]	70	90	74	38	36	41	41	43
Acid RSM [a]	67	90	70	29	24	30	37	32
Acid RSM + [b]	68	89	71	32	26	30	39	34
Pooled SE [c]	0.43	0.40	0.49	0.97	1.51	1.34	1.01	1.34
P (Acid) [d]	0.140	0.945	0.008	0.567	0.219	0.719	0.531	0.259
P (Enzyme)	0.017	0.374	0.037	<0.001	0.004	<0.001	0.005	0.107
P (Acid x enzyme)	0.082	0.087	0.366	0.008	0.020	0.035	0.048	0.193

[a] Unpr. RSM: unprocessed RSM; Acid RSM: acid-extruded RSM. [b] Including addition of commercial pectolytic enzymes (+). [c] Pooled standard error. [d] Differences among means with P<0.05 were considered to represent significant differences.

Twenty-four percent of the non-glucose polysaccharides (NGP) in the unprocessed RSM-diet was fermented (Table 5.3). The NGP-fermentability was calculated rather than NSP-fermentability, as excreta still contained residual starch (analyzed to be 2-3% (w/w)). In addition, glucosyl residues may also derive from produced microbial glucans, next to xyloglucan and cellulose originating from RSM. The NGP-fermentability mainly describes the ability of the broilers to ferment pectin and arabinoxylan. Addition of commercial pectolytic enzymes significantly improved NGP-fermentability to 38% (P<0.001; Table 5.3). This was mainly visible in the improved fermentability of arabinosyl residues (P<0.001) and xylosyl residues (P= 0.005). Though not significantly, in the acid-extruded RSM-diet NGP-fermentability numerically increased to 29% (P=0.567).

In the diet containing acid-extruded RSM+, NGP-fermentability improved to 32%. This increase is lower than when enzymes were added to unprocessed RSM. In NGP-fermentability an interaction was found between acid-extrusion and enzyme addition (P=0.008), but this can only be explained by the fact that enzyme addition can significantly improve NGP-fermentability from unprocessed RSM and not from acid-extruded RSM. Opposed to the hypothesis described in the introduction, apparently, acid-extrusion made the matrix less accessible for enzymes during *in vivo* fermentation rather than more accessible. Even though addition of commercial pectolytic enzymes improved NGP-fermentability of unprocessed RSM, still 62% remained unfermented in this study. These unfermented carbohydrate structures were analyzed in more detail.

The dry matter digestion followed the same trend as the NGP-fermentation. Protein-digestion decreased by acid-extrusion of RSM (P= 0.008), but was improved by addition of commercial enzymes (P=0.037). Fat-digestion was not affected by acid-extrusion and/or addition of commercial pectolytic enzymes (Table 5.3).

5.3.3 Distribution of unfermented carbohydrates

The focus of this paper was to characterize the NSP-structures remaining in excreta of broilers fed with diets containing unprocessed and acid-extruded RSM. Excreta contained 40-44% (w/w) carbohydrates and 30-32% (w/w) proteins. The carbohydrates present contained mainly glucosyl (43-54 mol%), uronyl (20-27 mol%), and arabinosyl residues (11-17 mol%) (Table 5.4). The carbohydrates present were characterized in detail after extraction from the excreta with water and 6M NaOH. Figure 5.1 shows the distribution of carbohydrates being fermented or unfermented. In addition, in Figure 5.1 the unfermented carbohydrates were further divided into a water-soluble fraction (WSS), alkali-soluble fractions (ASS), and the remaining residue (RES). In WSS, 16-18% (w/w) of the total amount of carbohydrates in the excreta was extracted (Table 5.4). The amount of water-soluble carbohydrates was similar for all excreta. Unexpectedly, in ASS from excreta from broilers fed unprocessed RSM, unprocessed RSM+, and acid-extruded RSM only 5-7% (w/w) of the water unextractable carbohydrates were recovered (Table 5.6). For excreta from broilers fed acid-extruded RSM+ this value was 19%.

It was calculated from the NSP in the WUS-fractions minus the NSP collected in the ASS- and RES-fractions that 52-58% (w/w) of the NSP present in WUS were extracted with alkali, but that 39-52% (w/w) of these NSP were not recovered in the ASS- and RES-fractions. This suggested that an important part of the water unextractable carbohydrates was released by alkali and having a rather low molecular mass (<14 kDa, cut-off value of the dialysis membrane).

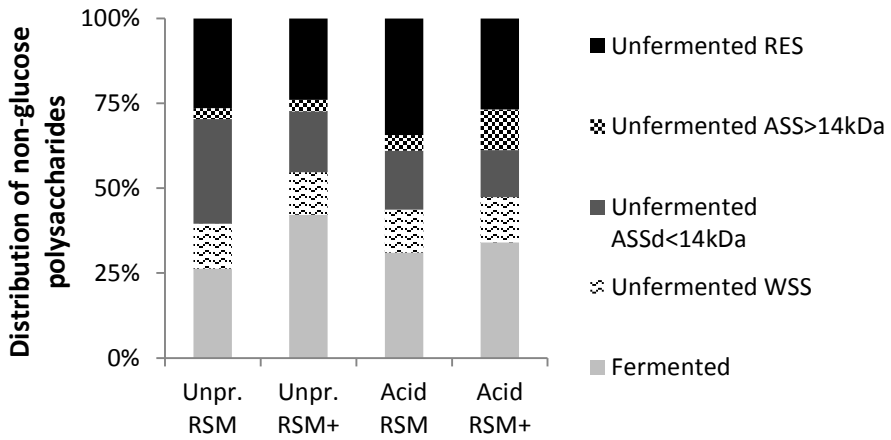

Figure 5.1. Division of non-glucose polysaccharides from rapeseed meal being either fermented or unfermented. The unfermented carbohydrates are further divided as water- (WSS), calculated alkali-solubles <14 kDa (ASSd) and alkali-solubles >14 kDa (ASS), and final residue (RES) of excreta from broilers fed unprocessed RSM (Unpr. RSM), unprocessed RSM with addition of commercial pectolytic enzymes (Unpr. RSM+), acid-extruded RSM (Acid RSM), and acid-extruded RSM with addition of commercial pectolytic enzymes (Acid RSM+).

These alkali extracted carbohydrates are referred to as ASSd <14 kDa. Acid-extrusion and/or addition of commercial pectolytic enzymes tended to decrease the relative amount of the released fraction (ASSd). The combination of acid-extrusion and addition of pectolytic enzymes increased the ASS-fraction and decreased the ASSd-fraction in the excreta, while WSS and RES were stable. This suggested that acid-extrusion increased rigidness of the NSP-matrix *in vivo*, which limited accessibility for the enzymes added.

A similar alkaline extraction of NSP from the unprocessed RSM added in the diet did not result in such a release of small carbohydrates (no further data shown). For this phenomenon observed, three possible hypotheses were considered. First, small carbohydrates (<14kDa), remaining from partly fermentation of the original RSM polysaccharide structures, were present in the excreta linked via ester-linkages. Ester-linkages to proteins and carbohydrates in RSM are reported to be present through sinapic, ferulic, and coumaric acid (*20*). Alkaline extraction will break such bonds, thereby solubilizing these partly fermented carbohydrates. This indicates that the broilers' microbiota was able to ferment part of the polysaccharides present, but full fermentation was hindered by ester-bonds. Second, 6M NaOH makes cellulose fibrils swell, as published

for bamboo fibers (*21*). Thereby non-covalently bound, e.g. by H-bonding, carbohydrate-structures can be released. Third, the small carbohydrates originating from NSP could be bound to the microbial cell surface proteins, as is a known digestive mechanism for Bacteroidetes (*22*), which make up 23-46% of the bacteria in the caeca of chicken (*23*). Again, alkali will then release these small carbohydrates. We believe the third theory is less likely, because of the relative high amounts of carbohydrates released. Also, first indications (HPSEC and HPAEC) about the size of these carbohydrates released (data not shown) show, besides very small carbohydrates, larger material than those expected to bind to cell surface proteins. Further research will be conducted to analyze the nature of these carbohydrates released. After water and alkaline extraction, still 32-37% (w/w) of the carbohydrates in the excreta of broilers fed unprocessed RSM, unprocessed RSM+, and acid-extruded RSM+ remained in the final residue (RES). This increased to 45% (w/w) in excreta of broilers fed acid-extruded RSM, again suggesting that the NSP-matrix became more rigid in these excreta. Arabinosyl residues were mainly alkali-soluble, probably originating from arabinan (Figure 5.2-A). Xylosyl residues were mainly alkali-soluble, probably originating from xylan or xyloglucan (Figure 5.2-B). Uronyl residues were both alkali-soluble and unextractable, probably originating from pectins, in different ways attached to the cell wall (Figure 5.2-C). Both acid-extrusion and addition of pectolytic enzymes did not affect the proportion of water-soluble arabinosyl, xylosyl, and uronyl residues. The increased proportion of NSP in the ASS-fraction in the excreta of broilers fed acid-extruded RSM+, was mainly caused by xylosyl residues. This indicated that more xylan retained in the ASS-fraction (>14 kDa), compared with the other treatments, suggesting that the rigidity of the NSP-matrix *in vivo* was increased. The proportion of released alkali-soluble (ASSd <14 kDa) unfermented arabinosyl residues was decreased in broilers fed unprocessed RSM+, in favor of the proportion of fermented arabinosyl residues. Compared with unprocessed RSM, in excreta from broilers fed acid-extruded RSM and acid-extruded RSM+ the arabinosyl residues in the ASS-fraction decreased in favor of arabinosyl residues in the RES, while fermentation was similar, again suggesting the NSP-matrix became more rigid, upon acid-extrusion.

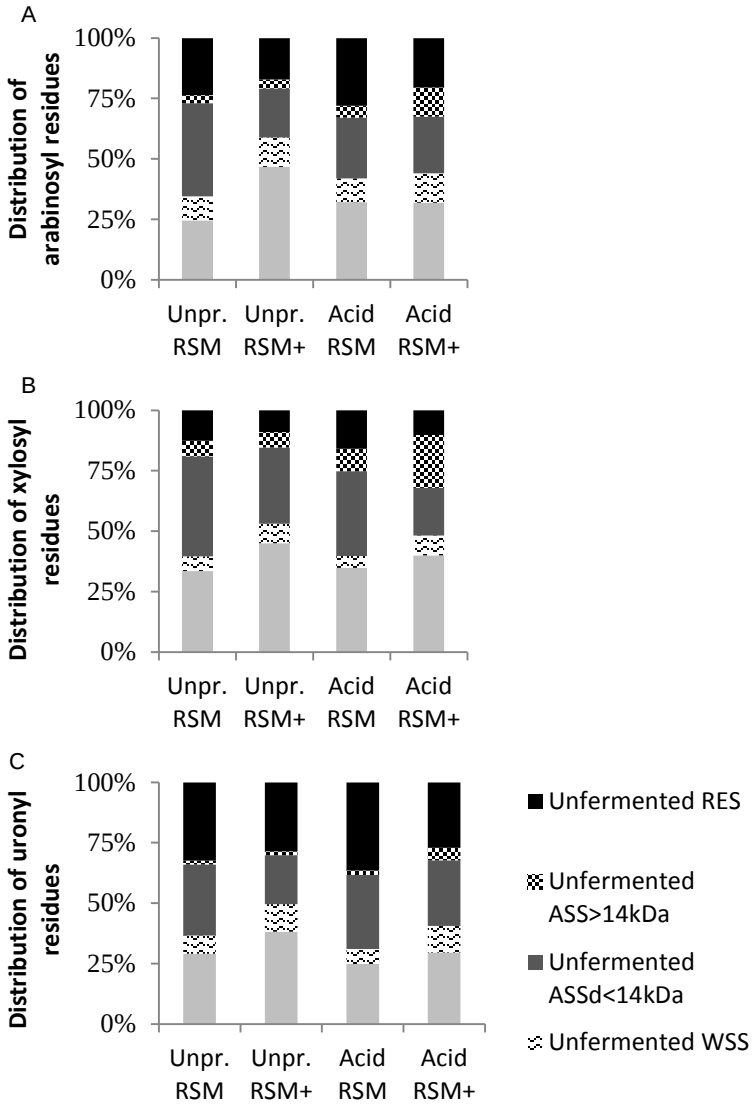

Figure 5.2. Distribution of arabinosyl (A), xylosyl (B), and uronyl (C) residues being fermented or unfermented. The unfermented residues are further divided as water-solubles (WSS), calculated alkali-solubles <14kDa (ASSd), alkali-solubles >14kDa (ASS), and final residue (RES) of excreta from broilers fed unprocessed RSM (Unpr. RSM), unprocessed RSM with addition of commercial pectolytic enzymes (Unpr. RSM+), acid-extruded RSM (Acid RSM), and acid-extruded RSM with addition of commercial pectolytic enzymes (Acid RSM+).

5.3.4 Characterization of unfermented water-soluble carbohydrate structures

The water-soluble fraction of excreta from broilers fed unprocessed RSM consisted of 47 mol% glucosyl, 15 mol% arabinosyl, 13 mol% uronyl, and 12 mol% galactosyl residues (Table 5.4). The molar composition of WSS of excreta from broilers fed the acid-extruded RSM-diet was similar. In the WSS of excreta from broilers fed unprocessed RSM+ and broilers fed acid-extruded RSM+, the molar proportion of glucosyl residues decreased and those of xylosyl and uronyl residues increased, compared with excreta from broilers fed unprocessed RSM. The significant improvement in fermentability of arabinosyl residues, even though the molar proportion of arabinosyl residues in the WSS-fraction was not affected by acid-extrusion or addition of pectolytic enzymes. Table 5.4 shows that (part of the) small saccharides, like fructose, raffinose, and stachyose (together 4-8% (w/w) of the carbohydrates in the WSS-fraction), present in the RSM in the diets (Table 5.2) remained in the excreta. As expected, saccharose was completely digested (*24*). Apart from the small saccharides mentioned, the constituent monosaccharide composition of larger carbohydrates was calculated to be 34-48 mol% glucosyl, 15-22 mol% uronyl, 13-14 mol% arabinosyl, and 13-14 mol% galactosyl residues (Table 5.4). This molar composition indicates the presence of (xylo-)glucans, homogalacturonan, arabinan, and galactan.

Table 5.4. Yield and constituent NSP carbohydrates (mol%) in water-soluble (WSS) and water unextractable (WUS) fractions of excreta after *in vivo* digestion in broilers fed diets containing unprocessed and acid-extruded RSM with or without addition of commercial pectolytic enzymes.

		Carbohydrate								
	Yield (%) [a]	Content (w/w%)	Yield (%)	Rha [b]	Ara	Xyl	Man	Gal	Glc	UA
Excreta Diet 1 (Unpr. RSM) [c]	100	**41**	100	**1**	**16**	**7**	**1**	**4**	**44**	**27**
WSS – total	38	17	16	1	15	7	5	12	47	13
Of which small saccharides [d]	*n.a. [e]*	*n.a.*	*8*	0	0	0	0	16	84	0
Of which polysaccharides	*n.a.*	*n.a.*	*92*	1	13	6	5	13	47	15
WUS	46	66	74	1	21	15	2	6	31	24
Recovery [f]	***84***		***90***							
Excreta Diet 2 (Unpr. RSM +) [g]	100	**44**	100	**1**	**11**	**7**	**1**	**4**	**53**	**23**
WSS – total	40	20	18	1	16	12	5	13	34	19
Of which small saccharides	*n.a.*	*n.a.*	*6*	0	0	0	0	16	84	0
Of which polysaccharides	*n.a.*	*n.a.*	*94*	1	14	10	5	13	35	22
WUS	54	59	73	1	14	18	2	6	38	21
Recovery	***94***		***91***							
Excreta Diet 3 (Acid RSM) [c]	100	**41**	100	**1**	**17**	**8**	**1**	**4**	**45**	**24**
WSS – total	39	17	17	1	15	7	5	13	46	13
Of which small saccharides	*n.a.*	*n.a.*	*4*	0	0	0	0	15	85	0
Of which polysaccharides	*n.a.*	*n.a.*	*96*	Tr. [h]	13	6	5	13	48	15
WUS	57	63	88	1	17	15	2	6	32	27
Recovery	***96***		***105***							
Excreta Diet 4 (Acid RSM +) [f]	100	**40**	100	**1**	**13**	**7**	**1**	**4**	**53**	**21**
WSS – total	38	19	18	1	16	12	5	13	34	19
Of which small saccharides	*n.a.*	*n.a.*	*4*	0	0	0	0	23	77	0
Of which polysaccharides	*n.a.*	*n.a.*	*96*	1	13	10	5	14	35	22
WUS	56	63	89	1	15	15	3	6	39	21
Recovery	***94***		***107***							

[a] Dry matter. [b] Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnose, Ara= arabinose, Xyl= xylose, Man= mannose, Gal= galactose, Glc= glucose, UA= uronic acid. [c] Unpr. RSM: unprocessed RSM; Acid RSM: acid-extruded RSM. [d] Small saccharides: fructose, saccharose, raffinose and stachyose. [e] n.a.: Not analyzed. [f] Recovery= recovery after water-extraction. [g] Including addition of commercial pectolytic enzymes (+). [h] Tr.: trace amounts.

5.3.4.1 Glycosidic linkage type analysis of WSS-fractions from excreta

The carbohydrate structures in WSS-fractions from excreta were further analyzed using glycosidic linkage type analysis (Table 5.5). Data obtained should be reviewed in a qualitative rather than in a quantitative manner due to the presence of uronic acids (24-36 mol% in polymeric WSS-fraction) (*25*). Also, it should be taken into account that in the protocol used, some small saccharides got dialyzed out together with excess of reagents. Nevertheless, the molar carbohydrate composition analyzed by glycosidic linkage type analysis (Table 5.5) and neutral carbohydrate composition analysis (Table 5.4) were quite similar.

Arabinosyl residues in WSS-fraction of excreta of broilers fed unprocessed RSM were mainly terminal linked and branched, both at the O2- and O3-position, indicating the presence of a branched arabinan, as found for *Brassica campestris* meal (*5*), but not for *Brassica napus* meal (*4*). In the same sample, xylosyl residues were found to be terminal and 1,2-linked, combined with terminal, 1,4-, and 1,4,6-linked glucosyl residues, indicating the presence of xyloglucan, which was also found in RSM itself (*4*). Xylosyl residues were also 1,4-linked, indicating the presence of a linear xylan, as was also present in RSM itself (*4*). Mannosyl residues were found to be 1,4,6-linked. Combined with terminal linked galactosyl residues, the latter indicates the presence of a galactomannan, as found in RSM itself (*4*). Galactosyl residues were found as 1,3-linked, indicating the presence of a linear galactan, as was also found in RSM itself (*4*). Some galactosyl residues were 1,6-linked, probably originating from raffinose and stachyose. Finally, a small amount of 1,6-linked glucosyl residues were found, most likely originating from the microbiota (*26*).

Arabinosyl residues in the WSS-fraction of excreta from broilers fed unprocessed RSM+ were only terminal and 1,5-linked. Hence, the addition of pectolytic enzymes decreased the branchiness of the arabinan considerably, as also was reflected in the disappearance of 1,2,5- and 1,3,5-linked arabinosyl residues. Enzymes were, thereby, helping in degrading arabinans, which is in line with the positive effect of enzyme addition on arabinose fermentability ($P<0.001$; Table 5.3). However, the molar proportion of arabinosyl residues in the WSS-fraction in the excreta did not decrease upon enzyme addition to RSM, while the molar proportion in the WUS did. Other glycosidic linkages were similar to those in WSS in excreta of broilers fed unprocessed RSM.

Glycosidic linkages present in the WSS-fraction of excreta from broilers fed acid-extruded RSM were very similar to those in the WSS of excreta from broilers fed unprocessed RSM.

Similar to the diet containing unprocessed RSM+, also in excreta from broilers fed acid-extruded RSM+ the branchiness of arabinans decreased.

Table 5.5. Glycosidic linkage composition (mol%) of water- (WSS) and alkali- (ASS) solubles of excreta after *in vivo* digestion in broilers fed diets containing unprocessed and acid-extruded RSM.

	WSS - Excreta				**ASS - Excreta**			
	Unpr. RSM [a]	Unpr. RSM+ [b]	Acid RSM[a]	Acid RSM+ [b]	Unpr. RSM[a]	Unpr. RSM+ [b]	Acid RSM [a]	Acid RSM+ [b]
t-Ara [c]	9	6	8	6	9	9	10	8
1,2-Ara		1					1	1
1,5-Ara	3	4	3	3	3	3	3	2
1,2,5-Ara	4		4					
1,3,5-Ara	1		1		1	1	1	
Total Ara	**17**	**11**	**16**	**9**	**13**	**13**	**15**	**11**
t-Xyl	2	5	2	6	10	10	9	9
1,2-Xyl	1	2	1	2	4	4	4	4
1,4-Xyl	1	2	1	2	10	10	9	9
1,3,4-Xyl					8	8	9	9
1,2,3,4-Xyl					3	5	3	5
Total Xyl	**4**	**9**	**4**	**10**	**35**	**37**	**34**	**36**
1,4-Man					6	8	8	6
1,4,6-Man	4	3	4	3	3	3	3	3
Total Man	**4**	**3**	**4**	**3**	**9**	**11**	**11**	**9**
t-Fuc	Tr	2	2	3	2	2	2	2
1,2,4-Fuc								
Total Fuc		**2**	**2**	**3**	**2**	**2**	**2**	**2**
t-Gal	5	9	5	10	7	7	6	7
1,2-Gal								
1,3-Gal	4	5	4	5				
1,6-Gal	2	2	2	2				
Total Gal	**11**	**16**	**11**	**17**	**7**	**7**	**6**	**7**
t-Glc	15	13	12	11				
1,4-Glc	42	37	43	36	18	18	18	19
1,6-Glc	2	1	3	5				
1,4,6-Glc	5	8	5	6	16	12	14	16
Total Glc	**64**	**59**	**63**	**58**	**34**	**30**	**32**	**35**
T/Br [d]	**2.29**	**3.52**	**2.04**	**3.91**	**0.79**	**0.77**	**0.84**	**0.70**

[a] Unpr. RSM: unprocessed RSM; Acid RSM: acid-extruded RSM. [b] Including addition of commercial pectolytic enzymes (+). [c] t= terminal linked. [d] Ratio between terminal linked and branched residues.

5.3.4.2 Enzymatic fingerprinting of WSS-fractions from excreta

Using pure and well-characterized enzymes, the polymeric carbohydrate structures indicated by glycosidic linkage type analysis can be confirmed.

The water-soluble arabinan, which accounted for ~13% (w/w) of the total carbohydrate content in WSS, was hydrolyzed using exo- and endo-arabinanases (Supplemental figure 5.1). Only 1-7% of arabinosyl content of the WSS-fraction could be released as arabinose DP 1-2. Apparently, the arabinan present is not accessible by the arabinanases used. This can be the result of the high branchiness of arabinan structures (unprocessed and acid-extruded RSM-diets) or the result of the presence of mainly terminal arabinosyl residues linked to other structures than arabinan, e.g. linked to galactan or xylan (diets containing unprocessed RSM+ and acid-extruded RSM+), which were not substrates for these arabinanases.

5.3.5 Characterization of unfermented water unextractable carbohydrate structures

Water unextractable solids (WUS) from the excreta of broilers fed unprocessed and acid-extruded RSM, contained mainly 31 mol% glucosyl, 24 mol% uronyl, 21 mol% arabinosyl, and 15 mol% xylosyl residues (Table 5.6). In the WUS of excreta from broilers fed unprocessed RSM+ and broilers fed acid-extruded RSM+, arabinosyl residues decreased and glucosyl residues increased compared with excreta from broilers fed unprocessed RSM.

The analyzed alkali-soluble fractions (ASS>14 kDa) in all the excreta samples were rather similar and contained mainly 32-35 mol% xylosyl, 24-26 mol% glucosyl, and 16-20 mol% arabinosyl residues (Table 5.6). As discussed in Section 3.3, 39-52% of the unextractable carbohydrates from excreta was calculated to be released by 6M NaOH, but not recovered in the ASS- or RES-fractions. The molar constituent monosaccharide composition was calculated to be: 25-37 mol% glucosyl, 24-36 mol% uronyl, 11-19 mol% xylosyl, 11-17 mol% arabinosyl, 6-8 mol% galactosyl, and 3 mol% mannosyl residues. The calculated release of glucosyl- and uronyl-residues in ASSd was relatively high compared with the other constituent monosaccharides. The released glucosyl-residues probably originate from (xylo)glucans previously bound to cellulose by H-bonding, as previously suggested (*27*). The released uronyl-residues maybe originate from pectins that were ester-linked in

Table 5.6. Yield and constituent NSP carbohydrates (mol%) alkali-solubles > 14kDa (ASS), alkali-solubles < 14kDa (ASSd) and RES of excreta after *in vivo* digestion in broilers fed diets containing unprocessed and acid-extruded RSM.

		Carbohydrate								
	Yield (%) [a]	Content (%w/w)	Yield (%)	Rha [b]	Ara	Xyl	Man	Gal	Glc	UA
Excreta diet 1 (Unpr. RSM) [c]										
WUS	100	**66**	100	**1**	**21**	**15**	**2**	**6**	**31**	**24**
ASS	4	78	5	tr. [d]	20	32	5	10	24	9
ASSd (<14kDa) [e]		n.a. [f]		1	17	14	3	9	29	27
RES	46	48	43	1	18	7	1	4	40	29
Recovery [g]	***50***		***48***							
Excreta diet 2 (Unpr. RSM +) [h]										
WUS	100	**59**	100	**1**	**14**	**18**	**2**	**6**	**38**	**21**
ASS	6	70	7	tr.	17	35	7	9	24	8
ASSd (<14kDa) [e]		n.a.		2	11	20	3	8	32	24
RES	64	59	48	1	11	7	1	4	53	23
Recovery	***70***		***55***							
Excreta diet 3 (Acid RSM) [c]										
WUS	100	**63**	100	**1**	**17**	**15**	**2**	**6**	**32**	**27**
ASS	5	89	7	tr.	20	32	5	10	24	9
ASSd (<14kDa) [e]		n.a.		2	13	14	3	8	25	35
RES	57	72	51	1	17	8	1	4	45	24
Recovery	***62***		***58***							
Excreta diet 4 (Acid RSM +) [h]										
WUS	100	**63**	100	**1**	**15**	**15**	**3**	**6**	**39**	**21**
ASS	15	79	19	tr.	16	34	5	10	26	9
ASSd (<14kDa) [e]		n.a.		1	11	11	3	7	37	30
RES	77	59	42	1	13	7	1	4	53	21
Recovery	***92***		***61***							

[a] Dry matter. [b] Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnose, Ara= arabinose, Xyl= xylose, Man= mannose, Gal= galactose, Glc= glucose, UA= uronic acid. [c] Unpr. RSM: unprocessed RSM; Acid RSM: acid-extruded RSM. [d] tr: Trace amounts. [e] Molar composition calculated from "loss" of alkali-soluble carbohydrates upon dialysis. [f] na: Not analyzed. [g] Recovery= recovery from WUS after alkaline extraction. [h] Including addition of commercial pectolytic enzymes (+).

the cell wall material, which has been hypothesized to occur via the acid-group of galacturonic acid (*28*).

5.3.5.1 Glycosidic linkage type analysis of ASS-fractions from excreta

Polymeric carbohydrate structures in the ASS-fraction >14 kDa were analyzed using glycosidic linkage type analysis and enzymatic fingerprinting (Section 5.3.5.2). Also here, data obtained should be reviewed in a qualitative rather than in a quantitative due to the presence of uronic acids (*25*). The results of the ASS-fraction of excreta from broilers fed unprocessed RSM showed 1,4-, 1,3,4-, and 1,2,3,4-linked xylosyl residues, indicating the presence of xylan branched either at the O3- or both at O2- and O3-positions (Table 5.5). Also terminal and 1,2-linked xylosyl residues were found. Combined with the 1,4- and 1,4,6-linked glucosyl residues found, this indicates the presence of xyloglucan. Comparing the structures in ASS-fraction of excreta with the ASS-fraction of undigested, unprocessed RSM, O2- and O3-branched xylan were found, while they were not found in RSM itself (*4*). In the excreta, they probably originate from maize present in the diets (Table 5.1), which is known to contain highly branched arabinoxylan-structures (*19*). Mannosyl residues were both 1,4- and 1,4,6-linked. Combined with terminal linked galactosyl residues, the latter indicates the presence of galactomannan, as was present in RSM itself (*4*).

The glycosidic linkages found in the ASS-fractions of excreta from broilers fed all four diets were similar. Apparently, the effect of acid-extrusion and enzyme addition only had an effect on the proportion of alkali-soluble carbohydrate, while there was no selective effect of acid-extrusion and addition of pectolytic enzymes on the structure of alkali-soluble carbohydrates analyzed.

5.3.5.2 Enzymatic fingerprinting of ASS-fractions from excreta

The xylan-oligomers formed after incubation with a pure endo-xylanase, as analyzed by MALDI-TOF-MS (Supplemental table 5.1), were present with and without glucuronic acid moieties attached, resulting from glucuronoxylans present. The xyloglucan-oligomers analyzed after incubation with xyloglucan-specific glucanase were XXXG or XSGG, XXLF or LSGG, XXFG and XLFG, indicating the presence of XXGG- and XXXG-type xyloglucan with decorations of arabinosyl, galactosyl and fucosyl residues, as were also found in RSM itself (*4, 29*).

As for the WSS-fraction, in the ASS-fraction, degradation of arabinan in the ASS-fraction was measured. Similar to the arabinan in the WSS-fraction, only 0-8% of arabinosyl content of the ASS-fraction was released as arabinose DP 1 and 2 (data not shown). Probably arabinosyl residues in the ASS-fraction were present as arabinoxylan originating from maize in the diet (Table 5.1), as suggested by the high amount of terminal linked arabinosyl and the highly branched xylosyl residues found using glycosidic linkage type analysis (Section 5.3.5.1).

Besides glucuronoxylan, xyloglucan, and arabinan, HPSEC-analysis of enzyme-digests of the ASS-fraction of excreta from broilers fed untreated RSM showed that endo-polygalacturonase and rhamnogalacturonan hydrolase were active towards the ASS-fractions (data not shown), confirming the presence of homogalacturonan and rhamnogalacturonan.

The degradation of alkali-soluble polysaccharides, as analyzed by HPSEC (data not shown) and MALDI-TOF MS (Supplemental table 5.1), in excreta from broilers fed all four diets was similar.

5.3.5.3 Unextractable carbohydrates

The unextractable residual fraction (RES) from excreta from broilers fed unprocessed RSM contained mainly 43 mol% glucosyl, 27 mol% uronyl, and 16 mol% arabinosyl residues (Table 5.6). This molar composition indicates the presence of cellulose and tightly bound pectins (*30, 31*). As for WSS, also the molar composition of RES of excreta from broilers fed the acid-extruded RSM-diet was similar to unprocessed RSM. But, in the RES of excreta from broilers fed unprocessed RSM+ and broilers fed acid-extruded RSM+, the molar proportion of arabinosyl residues decreased and glucosyl residues increased compared with unprocessed RSM. Polymeric carbohydrate structures could not be analyzed using glycosidic linkage type analysis due to their insolubility in DMSO. Nevertheless, using enzymatic fingerprinting some carbohydrate structures were elucidated (Supplemental table 5.1). Similar carbohydrate-structures were found in excreta from broilers fed unprocessed RSM+, acid-extruded RSM and acid-extruded RSM+.

In conclusion, NGP-fermentability of unprocessed RSM significantly improved from 24% to 38% after enzyme addition to the diet, mostly caused by an increase in fermentability of arabinosyl and xylosyl residues. Acid-extrusion was not able to improve NSP-digestibility significantly. Probably acid-extrusion resulted in a more rigid NSP-matrix *in vivo*, which also decreased the accessibility for pectolytic enzymes added in the diet.

In addition, our study indicated that during alkaline extraction of the WUS from excreta, half of the carbohydrates from excreta were not recovered in ASS and RES. Apparently, these unrecovered carbohydrates were present in the WUS via H-bonding or ester-linkages within the cellulose-lignin network. These linkages hindered complete fermentation.

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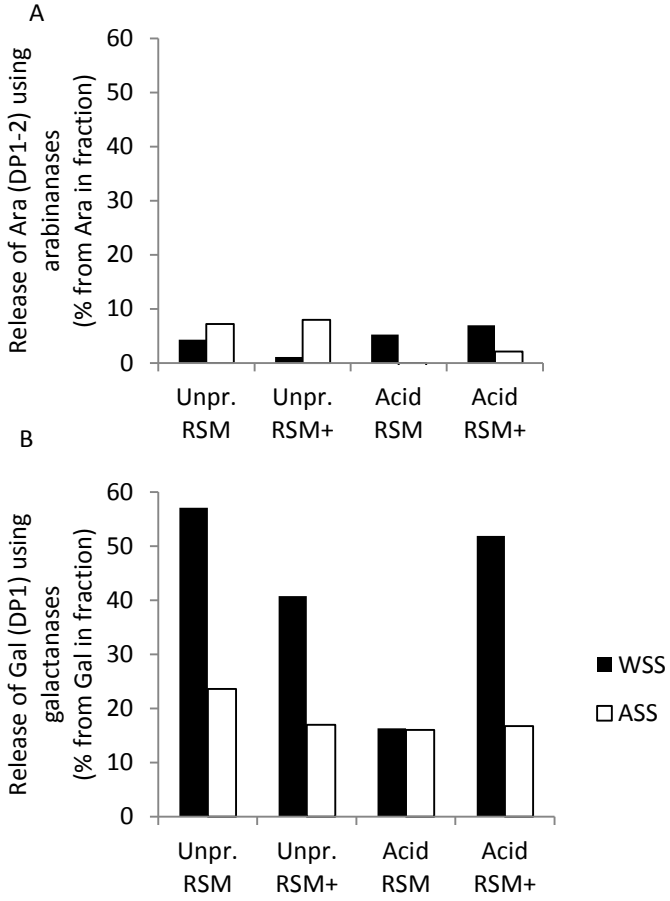
Supplemental table 5.1. Presence of oligosaccharide fragments after enzymatic fingerprinting of alkali- (ASS) solubles and final residue (RES) of excreta after *in vivo* digestion in broilers fed diets containing unprocessed and acid-extruded RSM (Hex=hexose, Pen= pentose, UA= uronic acid, OMe= 4-O-methylgroup).

	Unpr. RSM [a]		Unpr. RSM+ [b]		Acid RSM [a]		Acid RSM+ [b]	
Structure	ASS	RES	ASS	RES	ASS	RES	ASS	RES
Using arabinanases:								
$(Pen)_n$ (max. n) [b]	x (10)	-	x (10)	-	x (6)	-	-	-
551+$(Pen)_n$ (max. n)	-	x (9)	-	x (7)	-	x (9)	-	x (8)
Using endo-xylanase:								
$(Pen)_n$ (max. n)	x (6)	-	x (6)	-	x (6)	-	x (6)	-
$(Pen)_n$-UA-Ome (max. n)	x (6)	-	x (6)	-	x (6)	-	x (6)	-
Using polygalacturonase:								
$(UA)_n$ (max. n)	x (7)	-	x (7)	-	x (7)	-	x (6)	-
Using XG-specific glucanase:								
XXXG/XSGG, XXLF/LSGG, XXFG, XLFG [c]	x	-	x	-	x	-	x	-
Using cellulase:								
$(Hex)_n$ (max. n)	x (7)	-	x (7)	-	x (7)	-	x (7)	-
XSGG, LSGG, XXFG, XLFG [c]	x	-	x	-	+	-	X	-
$(Pen)_n$-Hex-UA (max. n)	-	x (8)	-	-	-	x (9)	-	x (9)

[a] Unpr. RSM: unprocessed RSM; Acid RSM: acid-extruded RSM. [b] Including addition of commercial pectolytic enzymes (+).
[b] Oligosaccharides of DP_n. [c] Nomenclature of xyloglucan-oligomers according to Fry et al. (*2*).



Supplemental figure 5.1. Release of arabinose (DP1-2) (A) and galactose (DP1) (B) after addition of endo- and exo-arabinanase, and beta-galactosidase and endo-galactanase, respectively, from the water- (WSS) and alkali- (ASS) solubles of excreta after *in vivo* digestion in broilers fed diets containing unprocessed (Unpr. RSM), unprocessed RSM with addition of commercial pectolytic enzymes (Unpr. RSM+), acid-extruded RSM (Acid RSM), and acid-extruded RSM with addition of commercial pectolytic enzymes (Acid RSM+).

Chapter 6

Fate of non-starch polysaccharides from (processed) rapeseed (*Brassica napus*) meal in the pigs' digestive tract

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Abstract

Unprocessed and acid-extruded rapeseed meal (RSM) was fed to pigs as the only source of non-starch polysaccharides (NSP) and protein. Fermentation of carbohydrate structures was followed in the digestive tract. Between ileum and end colon, constituent monosaccharides were gradually fermented by pigs. For the two feeds, 22% of total NSP was cumulatively fermented in the terminal ileum and around 70% total tract. Unfermented fecal carbohydrate structures were mainly rhamnogalacturonan, (branched) arabinan, XXXG-type xyloglucan, linear xylan, galactomannan, and cellulose. Nearly all water-soluble NSP were fermented. Acid-extrusion seemed to increase rigidness of the NSP-matrix *in vivo*, since the proportion of ileal water-soluble carbohydrates decreased and unfermented unextractable carbohydrates increased, while ileal NSP-fermentability remained the same. During alkaline extraction of the feces, 35-54% (w/w) of the insoluble carbohydrates was released as uronyl-rich carbohydrates, probably originally present via ester-linkages or hydrogen-bonding within the cellulose-lignin network. These linkages are expected to hinder complete NSP-fermentation.

6.1 Introduction

Rapeseed meal (RSM) is a by-product from the production of rapeseed oil. After pressing and solvent-extraction of oil, the meal is dried by solvent-toasting and used as animal feed for its high protein content (around 38% w/w (*1*)). Apart from protein, RSM is rich in non-starch polysaccharides (NSP; 16-22% w/w (*3*)), which potentially can be used as an energy source. The NSP present in RSM are represented by pectic polysaccharides (e.g homogalacturonan, rhamnogalacturonan, arabinan, and arabinogalactan), hemicelluloses (e.g. xyloglucan, glucuronoxylan, and galactomannan), and cellulose (*4, 5*).

Animals lack the necessary digestive enzymes that can degrade NSP. Nevertheless, in pigs, in the terminal ileum, in the cecum, and in the colon, gut microbiota ferment these carbohydrates into short chain fatty acids, lactate, gases, and microbial biomass. The extent of NSP-fermentation varies depending on individual NSP-structures. In general, pectins and hemicelluloses are better fermented than cellulose (*6*).

So far, RSM digestion studies have focused on protein digestibility. When polysaccharide fermentability was described, mostly neutral detergent, acid detergent and/or crude fiber contents were analyzed (*7, 8*). Or, in these digestion studies, the total NSP-content is calculated as dry matter minus the sum of crude protein, crude fat, ash, starch, and ethanol-soluble sugars(*9*), rather than based on analysis of each constituent carbohydrate present. In this way, fermentability of NSP from RSM was found to be around 58%, which is rather low compared with other NSP-rich ingredients, such as sugar beet pulp from which around 85% of the NSP is reported to be fermented by pigs (*9*). So, more energy is potentially available from RSM, if all the NSP present are fermented.

Since the unfermented carbohydrate structures have not been studied in detail so far, the rate-limiting step in NSP-utilization from RSM by pigs is not known. Therefore, in the present study, remaining carbohydrate structures in digesta of different parts of the pigs' digestive tract are studied. It has been shown in an *in vitro* assay, mimicking the ileum, that an acid-treatment, combined with addition of commercial pectolytic enzymes, worked best in solubilizing carbohydrates from RSM (*10*). Therefore, in this study, carbohydrate structures in digesta of pigs fed a diet containing untreated RSM are compared with a diet containing acid-treated RSM. Commercial pectolytic enzymes were not added in this study, since it is expected that enough enzymes are produced by the microbiota in the digestive tract (*11*). The aim of this study is to find the rate-limiting step in NSP-fermentation. Therefore, carbohydrate structures are monitored during

fermentation in pigs in detail. Also, it aims at determining whether acid-extrusion of RSM results in different unfermented carbohydrate-structures compared with unprocessed RSM.

6.2 Materials and methods

Chemicals were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA) or VWR International (Radnor, PA, USA), unless stated differently.

6.2.1 In vivo trial: animals and diets

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

6.2.1.1 Animals and pens

Twelve gilts (Topigs20xTalent) with body weight 20.8±2.4 kg were divided over two pens. Each pen was equipped with two drinking nipples and one feeding trough, which was long enough to allow simultaneous eating of all pigs. Temperature was maintained between 16°C and 23°C. The pigs were fed twice a day, 2.6 times their energy requirements for maintenance.

6.2.1.2 Experimental diets

The ingredient compositions of the diets are presented in Table 6.1. Rapeseed meal (Cargill, Antwerp, Belgium, 2011, supplied by Agrifirm, Apeldoorn, The Netherlands) was used as the only NSP- and protein-source in the diets. Diets were designed to meet the nutrient requirements of pigs according to CVB (*9*).

The first diet (Unprocessed RSM) contained 585 g/kg untreated RSM and the second diet contained 585 g/kg acid-extruded RSM (Acid-extruded RSM). Prior to extrusion, RSM, water (40% (w/w)), and maleic acid (2% (w/w)) were mixed using a paddle-mixer (Type F60, Halvor Forberg, Bygland, Norway). After 1 hour, acid-extrusion was performed in a double screw extruder (Baker-Perkins, Peterborough, UK) at 120˚C (product temperature at the die: 108˚C), feeding rate 33 kg/h, 250 rpm screw speed, and die size 6 mm.

Table 6.1. Ingredient composition of the diets containing RSM (g/kg as fed) and analyzed content of starch, protein, fat, and NSP (%w/w) and molar composition of NSP (mol%).

	Unprocessed RSM-diet	Acid-extruded RSM diet
Unprocessed rapeseed meal	585.5	
Acid-extruded rapeseed meal		585.5
Maize starch	269.4	269.4
Sugar	70	70
Vegetable oil	40	40
Premix [a]	10	10
Monocalcium phosphate	6	6
Limestone	5	5
Salt	4	4
$NaHCO_3$	3	3
$KHCO_3$	3	3
L-lysine HCl	3	3
L-threonine	0.6	0.6
L-tryptophan	0.3	0.3
Chromium oxide	0.22	0.22
Analyzed composition (w/w% dry matter)		
Protein	20	19
Starch	31	30
NSP [b]	26	24
Molar composition of NSP (mol%) [c]		
Rha	1	1
Ara	20	20
Xyl	9	8
Man	5	3
Gal	7	7
Glc	31	30
UA	27	31

[a] Mineral and vitamin premix provided the following per kg feed: vitamin A: 6,000 IU; vitamin D3: 1,200 IU; vitamin E: 40 mg; vitamin K3: 1.5 mg; vitamin B1: 1 mg; vitamin B2: 3 mg; vitamin B6: 1 mg; vitamin B12: 15 µg; niacin: 20 mg; D-panthothenic acid: 10 mg; choline chloride: 150 mg; folic acid: 1.2 mg; Fe: 80 mg ($FeSO_4 \cdot H_2O$); Cu: 15 mg ($CuSO_4 \cdot H_2O$); Mn: 30 mg (MnO); Zn: 50 mg ($ZnSO_4 \cdot H_2O$); Co: 0.2 mg ($CoSO_4 \cdot 7H_2O$); I: 0.7 mg (KI); Se: 0.2 mg (Na_2SeO_3). [b] NSP as extracted from the feed. [c] Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnose, Ara= arabinose, Xyl= xylose, Man= mannose, Gal= galactose, Glc= glucose, UA= uronic acid.

During a transition phase of 5 days, the feed was gradually changed from starter feed to the experimental feed. Pigs received diets in liquid form with a feed to water ratio of 1:3. An adaptation period of 14 days was followed by a collection period of 4 days, during which fecal samples were collected and pooled per pen.

6.2.1.3 Digesta collection

After the collection period (days 24 and 25 of the trial), animals were anesthetized and digesta samples were collected, after which animals were euthanized. Pigs were fed about 4h prior to section to ensure presence of fresh digesta in the terminal ileum.

Digesta were collected from terminal ileum (last 100 cm from ileocecal valve) and cecum. The colon was separated in three equal parts (proximal, mid and distal colon) from which the middle 50 cm were used to collect digesta. Samples were pooled per pen and immediately stored frozen (-20°C). Digestibility of protein and starch and fermentability of NSP and constituent monosaccharides in the different intestinal compartments was calculated using the following formula:

$$\text{Nutrient digestibility or fermentability} = 100 * \left(1 - \frac{[chromium]_{feed}}{[chromium]_{digesta}} * \frac{[nutrient]_{digesta}}{[nutrient]_{feed}}\right)$$

6.2.2 Extraction of water- and alkali-solubles from digesta and fecal samples

Unprocessed and acid-extruded RSM, and their corresponding digesta from terminal ileum, cecum, proximal, mid and distal colon, and fecal samples were freeze-dried and milled using a ball mill (Retsch MM2000, Haan, Germany). The milled samples (~2.5g) were extracted three times with 45 mL demineralized water during 1 hour at 40°C. After each extraction, the solubilized material was separated from the insoluble residue by filtration over Whatman 595½ filters (Whatman, Kent, UK). Water-soluble fractions were combined, freeze-dried, and denoted as water-soluble solids (WSS). Water unextractable solids (WUS; ~0.75g) obtained were further extracted twice using 6M NaOH including 20mM $NaBH_4$ at 4°C. After each extraction, material was centrifuged (48,400*g*, 20 min, 4°C) and the supernatant was further separated from the insoluble residue by filtration on

G2-glass filters. Alkali-soluble fractions were combined, neutralized using 6M HCl, dialyzed against demineralized water at 4˚C (Visking dialysis tubes, molecular mass cut-off 12-14kDa, pore diameter ca. 25Å, Serva, Heidelberg, Germany), freeze-dried, and denoted as alkali-soluble solids (ASS). The final residue was neutralized using 6M HCl, washed with water, freeze-dried, and denoted as residue (RES).

6.2.3 Enzymatic fingerprinting

WSS-, ASS-, and RES-fractions from ileal digesta and fecal samples (5mg/mL) were incubated with pure, well-characterized enzymes. The choice for specific enzymes to demonstrate the presence of specific polysaccharides was based on the carbohydrate composition of these fractions. The enzymes used were polygalacturonase, rhamnogalacturonan hydrolase, β-galactosidase, endo-galactanase, endo-arabinanase, exo-arabinanase, a xyloglucan specific endo-glucanase, and endo-xylanase I, as described previously (*4*). Pure enzymes were dosed at 0.1 µg enzyme-protein per mg substrate. Besides these pure enzymes, a commercial cellulase preparation (CellicCTec, Novozymes, Bagsvaerd, Denmark) was used and dosed at 20 µg enzyme-protein per mg substrate.
Incubations were performed in 10mM NaOAc buffer (pH 5.0) at 40˚C rotating 'head-over-tail' for 24 hours. All enzymes were inactivated by heating at 100˚C for 10 minutes. Digests were analyzed by HPSEC, HPAEC, and MALDI-TOF MS.

6.2.4 Analytical methods

Dry matter content was determined in duplicate by drying overnight in an oven at 103°C (WTC Binder, Tüttlingen, Germany).
Protein content (N*5.3 (*12*)) was determined in duplicate by the Dumas method (*13*) on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Interscience, Troy, NY, USA). Samples (~10 mg) were weighed into cups and directly analyzed. D-methionine was used for calibration.
Total starch content was determined enzymatically using the total starch assay procedure K-TSTA 04/2009 (Megazyme, Bray, Ireland).
Non-starch polysaccharide extraction from the feed was performed as described elsewhere (*11*). In short, the present starch was gelatinized and enzymatically degraded, after which NSP could be precipitated using acidified ethanol.

Neutral carbohydrate content and composition was determined in duplicate according to Englyst and Cummings (*14*). After a pretreatment with 72% (w/w) H_2SO_4 for 1h at 30°C, the samples were hydrolyzed in 1M H_2SO_4 at 100°C for 3h. Afterwards, the released monosaccharides were derivatized into their alditol acetates and analyzed by gas chromatography (Focus-GC, Thermo Scientific, Waltham, MA, USA). Inositol was used as internal standard.

Uronic acid content was determined in duplicate according to the automated colorimetric m-hydroxydiphenyl (Thermo Fisher Scientific) assay (*15*), including tetraborate, using an auto-analyzer (Skalar Analytical, Breda, The Netherlands). Galacturonic acid was used for calibration.

High Performance Size Exclusion Chromatography (HPSEC) was performed on an Ultimate 3000 System (Dionex, Sunnyvale, CA, USA) equipped with a set of four TSK-Gel superAW columns (Tosoh Bioscience, Tokyo, Japan) in series: guard column (6mm ID x 40mm) and separation columns 4000, 3000 and 2500 (6mm ID x 150mm). Samples (10µL) were eluted with aqueous 0.2M sodium nitrate for 25 minutes at 55˚C and at a flow rate of 0.6ml/min followed by refractive index detection (Shodex RI 101; Showa Denko K.K., Tokyo, Japan). Calibration was performed using pullulan standards of 180, 738 Da and 6, 12, 23, 47, and 112kDa. Enzyme digests were analyzed without prior dilution.

High Performance Anion Exchange Chromatography (HPAEC) was performed on an ICS-5000 System (Dionex) equipped with a CarboPac PA 1 column (2x250mm), and pulsed amperiometric detection. Elution was performed at a flow rate of 0.3 mL/min and a temperature of 40˚C. The elution conditions used for quantification of fructose, saccharose, raffinose, and stachyose in WSS were: 0-5 minutes isocratic 0.1M NaOH (prepared with 50% (w/v) NaOH solution, Boom BV, Meppel, The Netherlands), 5-15 minutes linear to 0.1M NaOAc in 0.1M NaOH, 15-25 minutes linear 0.1 to 0.3M NaOAc in 0.1M NaOH, isocratic for 5 minutes at 1M NaOAc in 0.1M NaOH, followed by 20 minutes isocratic at 0.1M NaOH. WSS-fractions (~5 mg/mL) were ten times diluted before analysis. Other elution conditions were used for the analysis of enzyme digests: 0-45 minutes linear from 0.1M NaOH to 0.7M NaOAc in 0.1M NaOH, isocratic for 5 minutes at 1M NaOAc in 0.1M NaOH, followed by 15 minutes isocratic at 0.1M NaOH. Enzyme digests were diluted ten times before analysis.

Matrix Assisted Laser-induced Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) was performed using an UltraFlextreme workstation (Bruker Daltonics,

Bremen, Germany) equipped with a Smartbeam II laser of 355nm and operated in the positive mode. After a delayed extraction time of 70 ns, the ions were accelerated to a kinetic energy of 25 kV and detected using a FlashDetector. The data were collected from averaging 200 laser shots, with the lowest laser energy needed to obtain sufficient spectra. External calibration was performed using maltodextrins (Paselli MD-20, AVEBE, Veendam, The Netherlands). Samples were desalted prior to analysis using AG 50W-X8 Resin (BioRad Laboratories, Hercules, CA, USA). 1µL desalted sample was mixed with 1µL matrix solution of 10mg/mL 2,5-dihydroxy-benzoic acid (Bruker Daltonics) in 50% (v/v) acetonitrile and dried under a stream of air.

Glycosidic linkage analysis was performed as described elsewhere (*16*). In short, polysaccharides were methylated, followed by hydrolysis with 2M TFA for 1 hour at 121°C and permethylated monomers were converted into their alditol acetates. The treatment with methyliodide was performed twice. The partially methylated alditol acetates were identified by GC-MS using a Trace GC coupled to a DSQ-II (both Thermo Scientific) equipped with a Restek RTX-35MS column (Restek, Bellefonte, PA, USA). A temperature gradient was applied from 120°C to 250°C in 52 minutes, proceeded by a hold time of 5 minutes at 250°C. MS detection of masses 50-450 *m/z* was performed.

Chromium (used as marker, added in the diets to enable calculation of nutrient digestibility) was analyzed by atomic absorption spectrometry after burning the samples at 250°C and incineration at 550°C, as described elsewhere (*17*).

6.3 Results and discussion

6.3.1 Characteristics of unprocessed and acid-extruded RSM

The RSM (Table 6.2; Unprocessed) was mainly composed of protein (33% (N*5.3) (w/w)) and carbohydrates (27% (w/w); including 25% (w/w) NSP). Other compounds in RSM are expected to be lignin, fat, and ash (*1*). RSM carbohydrates mainly contained glucosyl (40 mol%), arabinosyl (19 mol%), and uronyl residues (18 mol%) (Table 6.2).

The molar carbohydrate composition indicated the presence of cellulose, xyloglucan, arabinan, and homogalacturonan, as was described before for *Brassica napus* meal (*4*). The NSP-content and molar composition were similar to the batch characterized previously, but the NSP-content was lower compared with the batch used for our previous studies, where 36% (w/w) carbohydrate was found (*4*).

Table 6.2. Composition of unprocessed and acid-extruded rapeseed meal (g/100g dry matter).

	Unprocessed RSM	Acid-extruded RSM
Protein	33	30
Carbohydrate	27	35
Of which water-soluble saccharides (DP≤4) [a]	*7*	*12*
Of which water-soluble saccharides (DP>4) [a]	*19*	*17*
Of which water unextractable [a]	*74*	*71*
Others (lignin, ash, fat) [b]	40	35
Molar composition of carbohydrates [c]		
Rha	1	1
Ara	19	19
Xyl	8	8
Man	4	4
Gal	10	10
Glc	40	40
UA	18	18

[a] Percentage of water-soluble fructose, saccharose, raffinose, and stachyose (DP≤4) analyzed using HPAEC, other water-soluble saccharides (DP>4), and water unextractable carbohydrates from total carbohydrates.
[b] Calculated as dry matter minus protein and minus carbohydrates. [c] Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnosyl, Ara= arabinosyl, Xyl= xylosyl, Man= mannosyl, Gal= galactosyl, Glc= glucosyl, UA= uronyl.

The batch used for the *in vitro* study even contained 47% (w/w) carbohydrates, with the surplus mostly caused by extra uronyl residues (*10*). The carbohydrate content in RSM is variable and can differ between cultivars (*18*) and can be affected by treatment during oil extraction (*19*). The NSP-content of 25% (w/w) found in this study is in line with values reported previously (16-22% (w/w) (*3*)). Unexpectedly, the carbohydrate content was analyzed to be slightly higher and protein content slightly lower after acid-extrusion. This was not expected to be a result from acid-extrusion, since it was not seen in a previous study where RSM was treated similarly (*20*). It can be noted that taking a homogeneous sample from this large batch is difficult. The constituent monosaccharide composition was not affected by the processing performed (Table 6.2).

6.3.2 Animal performance

All 12 pigs stayed in good health through-out the study. The feed conversion ratio of pigs fed unprocessed RSM was 1.77 kg feed/kg body weight gain and was slightly improved to 1.66 kg feed/kg body weight gain by acid-extrusion of RSM. This is probably due to the

increased ileal protein-digestibility from 60% in unprocessed RSM to 69% in acid-extruded RSM. Pigs absorb proteins especially in the ileum, not in the cecum or colon (*21*).

6.3.3 In vivo fermentation of NSP from unprocessed and acid-extruded RSM

The digestibility and fermentability values were based on 6 animals per feed and were presented to be indicative rather than statistically analyzed (Table 6.3). In pigs fed a diet containing unprocessed RSM, 22% of the NSP was fermented in the ileum, 56% of the NSP was cumulatively fermented up to the cecum, and 68% fermented total tract (Table 6.3). Ileal NSP-fermentability from these RSM-rich diets was slightly lower compared with ileal NSP-fermentability of other diets, containing similar NSP, e.g. based on chicory forage and sugar beet pulp (*22*). However, total tract fermentability from the RSM-rich diets in this study was higher than expected from literature (*9*). Ileal NSP-fermentability was not influenced by acid-extrusion of RSM, while cecal NSP-fermentability of pigs fed acid-extruded RSM was slightly lower compared with pigs fed unprocessed RSM. Nevertheless, fecal fermentability was numerically increased to 72% for pigs fed acid-extruded RSM. The apparent decrease in cecal NSP-fermentability of acid-extruded RSM may result from selective digesta transport into the ceca. Selection may be based on solubility and particle size, but cecal digesta are only collected at one time point. In addition, the difference in cecal fermentability can be caused by a difference in passage rate, maybe affected by acid-extrusion. In the proximal colon NSP-fermentability for both diets are more similar (67-69%).

Next to total NSP-fermentability, also fermentation of individual constituent monosaccharides, being the building blocks of the NSP present, was analyzed. It can be seen (Table 6.3) that individual constituent monosaccharides were gradually fermented during passage through the digestive tract. Unexpectedly, ileal fermentability of arabinosyl, xylosyl, and uronyl residues decreased in pigs fed acid-extruded RSM compared with pigs fed unprocessed RSM, while fermentability of glucosyl residues increased. Although, the standard deviations are high and the results are only numerically different, this may suggest that acid-extrusion resulted in a more rigid NSP-matrix in the ileal digesta. Total tract uronyl residues were most difficult to ferment (51%), while arabinosyl (85%), xylosyl (79%) and glucosyl (74%)

Table 6.3. Apparent digestibility of protein and starch and fermentability of non-starch polysaccharides (NSP), arabinose (Ara), xylose (Xyl), glucose (Glc), and uronic acid (UA) in ileum, cecum, and proximal, mid and distal colon, and total tract in pooled samples from 12 pigs fed a diet containing unprocessed RSM (Unpr. RSM) or acid-extruded RSM (Acid RSM) as the only NSP- and protein-source.

	Unpr. RSM						Acid RSM					
	Ileum [a]	Cecum	Prox. colon	Mid colon	Distal colon	Total tract [a, b]	Ileum [a]	Cecum	Prox. colon	Mid colon	Distal colon	Total tract [a, b]
Protein	60±9.05	65	70	70	73	70±1.24	69±2.32	65	69	71	73	70±1.58
Starch	98±0.13	99	99	99	100	100±0.01	98±0.36	98	99	100	100	100±0.03
NSP	22±4.89	56	69	73	70	68±0.78	23±7.09	46	67	72	76	72±1.92
Ara	21±10.19	74	82	85	85	85±0.61	12±10.21	63	83	88	88	85±1.59
Xyl	25±13.78	61	77	80	80	79±0.85	3±8.91	50	75	82	85	82±1.76
Glc	37±4.86	60	75	80	75	74±1.19	43±8.01	47	67	73	79	75±2.14
UA	18±4.26	42	55	59	54	51±0.64	10±5.56	28	53	58	64	58±3.47

[a] Ileal digesta and feces were analyzed for pigs individually (n=6 per treatment), therefore average± standard error are presented. Content of other colon compartments were pooled prior to analysis. [b] Total tract sample collected at 4 time points.

showed a higher fermentability. This is different from previously described studies where pectins were assumed to be easily fermentable (*6*). It should be taken into account that a small amount of (partly digested) starch was still present (analyzed to be ≤1% (w/w)). Also, glucosyl residues in digesta and feces may derive from microbial glucans, next to xyloglucan and cellulose originating from RSM.

6.3.4 Distribution of unfermented carbohydrates

The ileal digesta mostly contained carbohydrates (52% (w/w)), next to proteins (15-19% w/w) (Table 6.4; Supplemental table 6.1). From the fecal dry matter still 30-33% (w/w) was carbohydrates and 23% (w/w) protein. Constituent monosaccharide compositions of digesta in ileum, cecum, colon, and feces are shown in Table 6.4. Since the molar monosaccharide compositions of the digesta in the different intestinal compartments were rather similar, it was again concluded that all constituent monosaccharides were gradually fermented during fermentation in ileum, cecum, and colon.

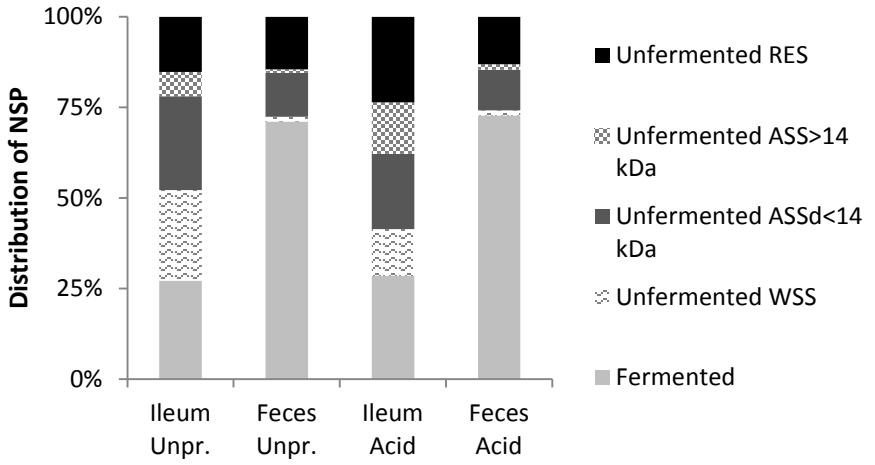

Figure 6.1. Distribution of NSP from rapeseed meal being digested or undigested. The undigested carbohydrates are further divided as water-solubles (WSS), calculated alkali-solubles <14kDa (ASSd), alkali-solubles >14kDa (ASS), and final residue (RES) of ileal digesta and feces of pigs fed unprocessed RSM (Unpr.) and acid-extruded RSM (Acid).

Figure 6.1 shows the distribution of carbohydrates originally present in the diet being fermented or unfermented. The unfermented carbohydrates were further divided into a water-soluble fraction (WSS), alkali-soluble fractions (ASS), and the remaining residue (RES). WSS constituted 26% (w/w) of the unfermented NSP in ileal digesta from pigs fed unprocessed RSM (Table 6.4). From the ileal digesta of pigs fed acid-extruded RSM, fewer carbohydrates were water-soluble (14% (w/w)), than from digesta of pigs fed unprocessed RSM. From the feces, only 4-5% of the unfermented NSP was extracted using water. So, nearly all water-soluble carbohydrates, as present in RSM itself and solubilized by microbial enzymes, were fermented in the cecum and colon (Figure 6.1), which was also observed for soy NSP (*23*).

Unexpectedly, only 8-10% (w/w) of the water unextractable carbohydrates in ileal digesta and feces from pigs fed unprocessed RSM was extracted in the corresponding ASS-fractions (Table 6.4). The yield of NSP from ileal digesta and feces from pigs fed acid-extruded RSM in the ASS-fraction was slightly higher (11-18% (w/w)), but still rather low. It was calculated from the NSP in the WUS-fractions minus the NSP collected in the ASS- and RES-fractions that 48-68% (w/w) of the NSP in WUS was extracted with alkali, but that 40-54% (w/w) of the NSP in WUS were not recovered in the ASS- and RES-fractions. This suggested that an important part of the water unextractable carbohydrates was released by alkali had a relatively low molecular mass (<14 kDa, cut-off value of the dialysis membrane). This alkali extracted carbohydrates are referred to as ASSd <14 kDa. A similar alkaline extraction of NSP from the unprocessed RSM added in the diet did not result in such a release of small carbohydrates (no further data shown). For this phenomenon observed, three possible explanations are considered. First, in the fecal samples, small, partly-fermented RSM carbohydrates were present linked via ester-linkages. Ester-linkages to proteins and carbohydrates in RSM are reported to be present through sinapic, ferulic, and coumaric acid (*24*). Alkaline extraction will break such bonds, thereby solubilizing these partly fermented carbohydrates. This explanation may indicate that the pigs' microbiota was able to degrade some of the polysaccharides present, but full degradation was hindered by ester-bonds. Second, 6M NaOH makes cellulose fibrils swell, as published for bamboo fibers, (*25*). Thereby, it releases low molecular weight non-covalently, e.g. by H-bonding, bound carbohydrate-structures. Third, small carbohydrates originating from RSM NSP can be bound to the microbial cell surface proteins mediated by a receptor, as is a known digestive mechanism for Bacteroidetes (*26*), which

Table 6.4. Carbohydrate content (w/w%), yield (%), and composition (mol%) in digesta, fecal samples, and fractions thereof, obtained after *in vivo* digestion in pigs fed diets containing unprocessed (Unpr. RSM) and acid-extruded RSM (Acid RSM).

	Rha [a]	Ara	Xyl	Man	Gal	Glc	UA	Carbohydrates	
								Content (%w/w) [b]	Yield (%)
Unpr. RSM									
Ileum	1	19	8	4	15	28	25	52	100
WSS [c]	*1*	*19*	*3*	*4*	*23*	*39*	*11*		26
ASS (>14 kDa) [d]	*1*	*24*	*19*	*3*	*11*	*20*	*22*		7
ASSd (<14 kDa) [e]	*1*	*20*	*9*	*5*	*10*	*13*	*42*		
RES [f]	*2*	*11*	*5*	*2*	*4*	*53*	*23*		16
Cecum	3	13	9	4	9	39	23	36	
Proximal colon	3	15	8	4	11	34	25	33	
Mid colon	3	15	8	4	12	29	29	30	
Distal colon	4	15	8	5	11	30	27	34	
Feces	1	14	8	4	11	35	27	33	100
WSS	*7*	*9*	*4*	*6*	*17*	*31*	*26*		4
ASS (>14 kDa)	*5*	*10*	*17*	*6*	*12*	*22*	*28*		3
ASSd (<14 kDa)	*2*	*9*	*5*	*5*	*9*	*16*	*54*		
RES	*2*	*8*	*4*	*3*	*5*	*48*	*30*		44
Acid RSM									
Ileum	1	21	10	4	9	22	33	52	100
WSS	*2*	*23*	*6*	*6*	*14*	*34*	*15*		14
ASS (>14 kDa)	*1*	*29*	*19*	*3*	*10*	*18*	*18*		15
ASSd (<14 kDa)	*1*	*21*	*10*	*4*	*11*	*7*	*46*		
RES	*2*	*10*	*4*	*2*	*4*	*56*	*22*		25
Cecum	2	16	10	4	9	35	24	40	
Proximal colon	1	17	9	4	11	31	27	34	
Mid colon	3	14	7	4	11	29	32	32	
Distal colon	5	14	7	6	11	30	27	28	
Feces	4	15	8	4	11	30	28	30	100
WSS	*9*	*8*	*4*	*6*	*17*	*30*	*26*		5
ASS (>14 kDa)	*4*	*18*	*15*	*5*	*13*	*18*	*27*		5
ASSd (<14 kDa)	*2*	*10*	*5*	*4*	*9*	*14*	*56*		
RES	*4*	*7*	*3*	*2*	*5*	*48*	*31*		46

[a] Molar composition presented as anhydrosugar moieties; Rha= rhamnose, Ara= arabinose, Xyl= xylose, Man= mannose, Gal= galactose, Glc= glucose, UA= uronic acid.
[b] Total carbohydrates based on dry matter (% w/w). [c] WSS: Water-soluble solids. [d] ASS: Alkali-soluble solids. [e] ASSd: Alkali-soluble solids in dialysate; numbers for ASSd <14 kDa were calculated as WUS minus ASS minus RES. [f] RES: Residue.

together with the phylum Firmicutes make up for >90% of the microbiota in pigs' large intestine (*27*). Again, alkali will then release these small partly fermented carbohydrates. We believe this third theory is less likely, because of the relative high amounts of released carbohydrates. Also, a similar extraction of a fecal sample from pigs fed a diet without RSM (samples from a study by Haenen and Souza da Silva, Division of Human Nutrition and Animal Nutrition Group, Wageningen University, to be published) did not result in such a release of carbohydrates. This indicated that the first two explanations are more likely. Further research will be conducted to analyze the nature of these carbohydrates released. In a previous *in vivo* trial where broilers were fed RSM-rich diets, also an important part (39-52% (w/w)) of the water unextractable carbohydrates in the excreta were extracted in alkali, but not recovered in the similarly prepared ASS- or RES-fractions from the excreta (*20*). Apparently, in both broilers and growing pigs fermentation of RSM NSP was hindered by the ester-linkages or H-bonding present.

After water and alkaline extraction, still 16% (w/w) of the ileal carbohydrates and 44% (w/w) of the fecal carbohydrates from pigs fed unprocessed RSM could not be extracted and remained in the residue (RES) (Table 6.4). In ileal digesta from pigs fed acid-extruded RSM, slightly more carbohydrates remained in the residue compared with unprocessed RSM. Combined with the decreased water-soluble carbohydrates, this indicated that, opposed to our hypothesis, *in vivo* the NSP-matrix became more rigid after acid-extrusion. In the feces the distribution of fractions is rather similar for pigs fed unprocessed RSM and acid-extruded RSM. Apparently, microbiota in the pigs' colon were hindered by this more rigid matrix. Distribution of fermented and unfermented arabinosyl, xylosyl, glucosyl, and uronyl residues over water-soluble, alkali-soluble, and residual fraction is shown in Figure 6.2. The yield of constituent monosaccharides in the ASSd (<14 kDa) fraction was again calculated from the amount of each constituent monosaccharide present in WUS minus the amount recovered in both the ASS- and RES-fractions. Arabinosyl residues were mainly water- and alkali-soluble, probably originating from arabinan (Figure 6.2-A). Xylosyl residues were mainly alkali-soluble, probably originating from xyloglucan and xylan (Figure 6.2-B). Glucosyl residues were both water-soluble, probably originating from glucans, and unextractable, probably originating from cellulose (Figure 6.2-C). Uronyl residues were mainly alkali-soluble, probably originating from pectins (Figure 6.2-D). The proportion of water-soluble arabinosyl residues in the ileal digesta decreased after acid-extrusion, in favor of the alkali-soluble fractions. The proportions of alkali-soluble xylosyl and uronyl

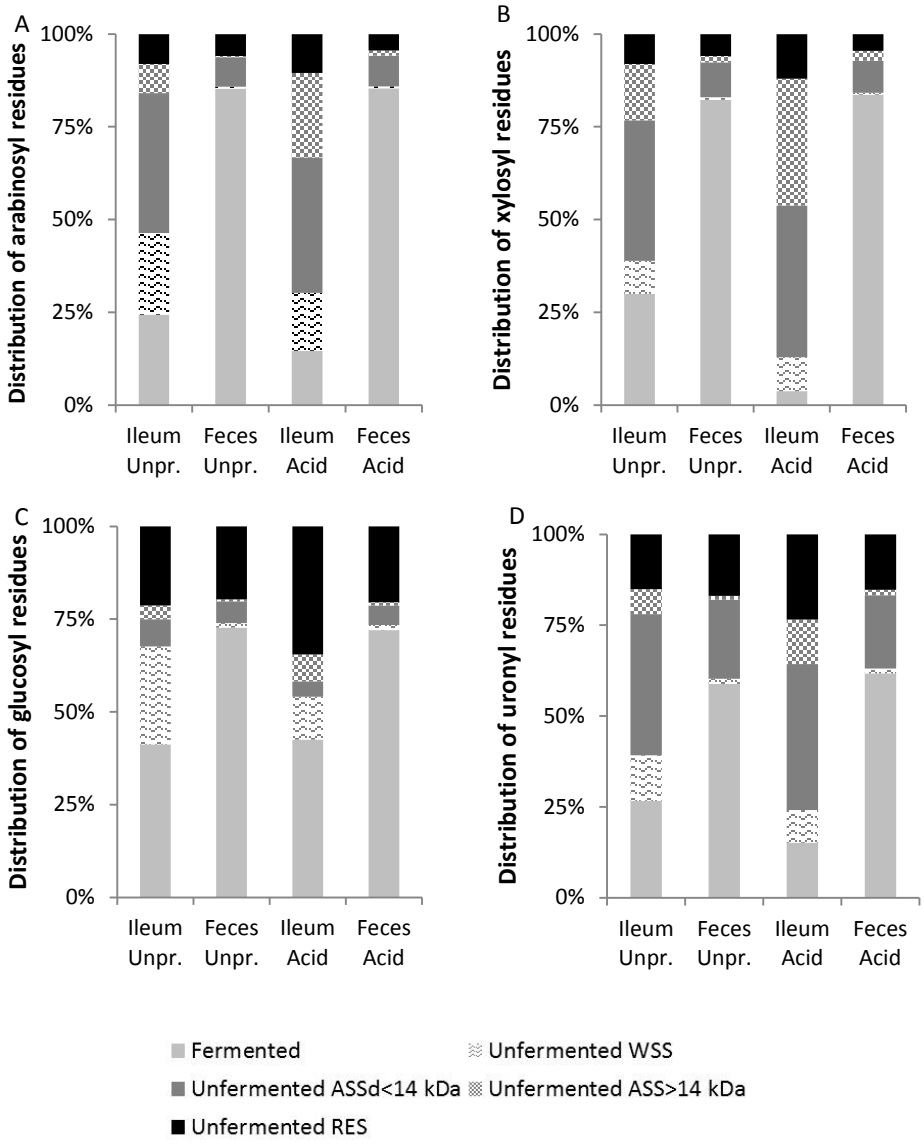

Figure 6.2. Distribution of arabinosyl (A), xylosyl (B), glucosyl (C), and uronyl (D) residues from rapeseed meal being digested or undigested. The undigested residues are further divided as water-solubles (WSS), calculated alkali-solubles <14kDa (ASSd), alkali-solubles >14kDa (ASS), and final residue (RES) of ileal digesta and feces of pigs fed unprocessed RSM (Unpr.) and acid-extruded RSM (Acid).

residues in the same ileal digesta increased at the expense of the fermented fraction. The proportion of water-soluble glucosyl residues in the same ileal digesta decreased in favor of the residue. Again, this suggested that acid-extrusion increased rigidity of the NSP-matrix in ileal digesta. This may explain the decreased ileal fermentabilities of the arabinosyl, xylosyl, and uronyl residues after acid-extrusion, compared with pigs fed unprocessed RSM.

6.3.5 Characterization of recalcitrant water-soluble carbohydrate structures

The carbohydrate contents of ileal and fecal WSS-fractions from pigs fed unprocessed and acid-extruded RSM are presented in Table 6.4. In the ileal WSS from pigs fed acid-extruded RSM, the molar proportion of arabinosyl was slightly higher and galactosyl residues was slightly lower than in fecal WSS from pigs fed unprocessed RSM. The molar compositions of fecal WSS from pigs fed unprocessed and acid-extruded RSM were rather similar and showed the presence of mainly glucosyl, uronyl, and galactosyl residues. Glycosidic linkage type analysis was needed to define the structures corresponding to this molar composition.

HPAEC-analysis (data not shown) of the WSS-fractions showed that small saccharides, being saccharose (1-3% (w/w) of the water-soluble carbohydrates), and fructose, raffinose, and stachyose (together 0-7% (w/w) of the water-soluble carbohydrates) were still present in digesta in ileum and cecum, but were almost completely digested and/or fermented in the colon. The total contents of these small saccharides in the unprocessed and acid-extruded RSM-samples are presented in Table 6.2.

6.3.5.1 Glycosidic linkage type analysis of ileal and fecal WSS-fractions

Carbohydrate structures in WSS-fractions were analyzed using glycosidic linkage type analysis (Table 6.5). It should be taken into account that in the protocol used some small saccharides got dialyzed out together with excess of reagents, possibly causing the high ratio between terminal and branched residues. Nevertheless, molar compositions analyzed by glycosidic linkage type analysis (Table 6.5) and neutral carbohydrate composition analysis (Table 6.4) were comparable.

Table 6.5. Glycosidic linkage composition (mol%) of water- (WSS) and alkali- (ASS) solubles of ileal digesta and feces after *in vivo* digestion in pigs fed diets containing unprocessed and acid-extruded RSM.

	WSS				ASS (>14 kDa)			
	Ileum		Feces		Ileum		Feces	
	Unpr. RSM	Acid RSM	Unpr. RSM	Acid RSM	Unpr. RSM	Unpr. RSM	Unpr. RSM	Unpr. RSM
t-Rha [a]			3	3				
1,2-Rha			2	3				
Total Rha			**5**	**6**				
t-Ara	9	18	4	3	15	14	4	9
1,2-Ara			1					
1,5-Ara	4	7	9	9	7	8	3	9
1,2,5-Ara	7	1			3	4	4	5
1,3,5-Ara	2	3			5	6		
1,2,3,5-Ara	12	4	4	5	9	4		
Total Ara	**34**	**33**	**18**	**17**	**39**	**36**	**11**	**23**
t-Xyl	1	4	2	2	11	8	6	6
1,2-Xyl	Tr [b]	1	1	1	5	4	4	4
1,4-Xyl	1	1	3	3	5	6	17	13
1,2,3,4-Xyl					2	1		
Total Xyl	**2**	**6**	**6**	**6**	**23**	**19**	**27**	**23**
1,4,6-Man			2	2			6	6
Total Man			**2**	**2**			**6**	**6**
t-Fuc	1	3	2	2				
1,2,4-Fuc								
Total Fuc	**1**	**3**	**2**	**2**				
t-Gal	14	7	9	8	5	4	8	8
1,2-Gal	2	3		4		5		
1,4-Gal				2	2	2	8	8
1,6-Gal	13	4	4	3				
Total Gal	**29**	**14**	**13**	**17**	**7**	**11**	**16**	**16**
t-Glc	14	12	14	11				
1,2-Glc	3			3				
1,3-Glc	2	3	5	6				
1,4-Glc	8	15	25	21	12	13	25	22
1,6-Glc	5	8	4	4				
1,3,4-Glc		2	3	2				
1,4,6-Glc	2	4	3	4	19	21	15	10
Total Glc	**34**	**44**	**54**	**50**	**31**	**34**	**40**	**32**
T/B [c]	**1.13**	**2.68**	**2.56**	**1.52**	**0.61**	**0.64**	**1.28**	**1.10**

[a] t: terminal. [b] Tr: trace amounts. [c] T/B: ratio terminally linked residues: branching points.

In the ileal WSS-fraction from pigs fed unprocessed RSM, arabinosyl residues were mainly 1,2,3,5- and terminal linked, next to 1,5-, 1,2,5-, and 1,3,5-linked arabinosyl residues. This indicates the presence of a highly branched arabinan with branches at the O2- and/or O3-position, as found for *Brassica campestris* meal (*5*), but not for *Brassica napus* meal (*4*). Galactosyl residues in the ileal WSS-fraction were found to be mainly terminal and 1,6-linked, probably originating from raffinose and stachyose. In the same sample, glucosyl residues were mainly present as terminal and 1,4-linked residues and some 1,4,6-linked residues, indicating the presence of some undigested (water-soluble) starch (analyzed to be 1% w/w), and possibly xyloglucan. Also smaller proportions of 1,2-, 1,3-, and 1,6- linked glucosyl residues were found, possibly originating from microbiota (*28*), since they were not found in RSM itself (*5*). After acid-extrusion of RSM, a lower proportion of 1,2,3,5-linked and a higher proportion of terminal linked arabinosyl residues was found in the WSS of corresponding ileal digesta compared with unprocessed RSM. This meant that the water-soluble arabinan was less branched compared with those found in ileal digesta of pigs fed unprocessed RSM. Most likely acid-extrusion released some side-groups from arabinan. Also in the *in vitro* study (*10*), arabinosyl residues were most affected by acid treatment, since arabinosyl residues are most acid-labile. The linkages of galactosyl residues in WSS of ileal digesta were not affected by acid-extrusion. Relatively more glucosyl residues in the ileal WSS were 1,4- and 1,4,6-linked compared with ileal WSS from pigs fed unprocessed RSM. Combined with the higher ratio of terminal linked xylosyl and fucosyl residues, this indicated the presence of xyloglucan in the WSS-fraction. Xyloglucan and cellulose can be tightly bound by hydrogen bonds (*29*) and most likely acid-extrusion disrupted some of these bonds and released xyloglucan into WSS. Such an acid-aided solubilization of xyloglucan was reported before for adzuki beans (*30*).

Despite the fact that in the fecal WSS-fraction of pigs fed unprocessed and acid-extruded RSM a relatively low amount of the carbohydrates was collected (Figure 6.1), the glycosidic linkages of the NSP present were still analyzed (Table 6.5). Mainly rhamnogalacturonan, arabinan, galactomannan, and xyloglucan were present, as were present in RSM itself (*4*). Branchiness of the fecal water-soluble arabinan decreased compared with the ileal water-soluble arabinan, implying that microbial enzymes present in the colon had debranching activity. Rhamnogalacturonan was not expected to be water-soluble, because in RSM itself these structures remained in the RES (*4*). Probably, the microbial enzymes present were able to solubilize rhamnogalacturonan.

6.3.5.2 Enzymatic fingerprinting of ileal WSS-fractions

The water-soluble arabinan, which was 15-20% (w/w) of the total carbohydrate content in the ileal WSS, was hydrolyzed using endo- and exo-arabinanases (Supplemental figure 6.1). From both ileal WSS from pigs fed unprocessed RSM and acid-extruded RSM, only 10-11% of the arabinosyl residues present could be released as arabinose DP 1-2 by the enzymes used. For the ileal water-soluble arabinan found in pigs fed unprocessed RSM these results confirmed the data obtained from glycosidic linkage type analysis. Together they pointed at the presence of an arabinan with substitutions at the O2- and the O3-position, which hindered degradation by endo-arabinanase and exo-arabinofuranosidase. For the water-soluble arabinan found in ileal digesta of pigs fed acid-extruded RSM, glycosidic linkage type analysis pointed at a less branched structure than the water-soluble arabinan in ileal digesta of pigs fed unprocessed RSM. Still, degradability by endo-arabinanase and exo-arabinofuranosidase was rather low. This suggested that the arabinan present was still too branched for the enzymes to degrade. The increase in terminal-linked arabinosyl residues, as observed in Table 6.5, possibly derived from arabinoxylan or arabinogalactan. Arabinan degradation in fecal WSS was not quantified, since this WSS-fraction is rather small (Figure 6.1). All NSP-structures present in the various WSS-fractions analyzed are summarized in Supplemental table 6.3.

6.3.6 Characterization of recalcitrant water unextractable carbohydrate structures

The water unextractable solids (WUS) from the digesta and feces mainly contained glucosyl (37-43 mol%), uronyl (19-40 mol%), and arabinosyl residues (14-24 mol%). To enable characterization of this fraction, WUS was further extracted using 6M NaOH (alkali-soluble solids, ASS).

From ileal digesta from pigs fed unprocessed RSM only 7% (w/w) of the unfermented carbohydrates was retained as alkali-soluble (ASS), while from ileal digesta from pigs fed acid-extruded RSM, 14% (w/w) of the unfermented carbohydrates were recovered in the ASS. The constituent monosaccharide composition of the ASS of ileal digesta and feces pointed at the presence of arabinan, xyloglucan, and pectic polysaccharides (Table 6.4).

As discussed in Section 6.3.4, 40-54% (w/w) of the water unextractable carbohydrates from ileal digesta and feces was released by 6M NaOH, but not recovered as ASS or RES.

The molar constituent monosaccharide composition of these fractions (ASSd<14 kDa) were calculated (Table 6.4). All ASSd fractions (<14 kDa) were calculated to be composed of 42-54 mol% uronyl residues. This was around twice the molar proportion of uronyl residues in the recovered ASS-fractions (>14 kDa).

6.3.6.1 Glycosidic linkage type analysis of ileal and fecal ASS-fractions

Carbohydrate structures in the ASS-fractions (larger than 14kDa) were analyzed using glycosidic linkage type analysis (Table 6.5). Data obtained from glycosidic linkage type analysis of the fractions should be reviewed in a qualitative way instead of quantitative due to incomplete DMSO-solubility and the presence of uronic acids (*31*).

The results indicated the presence of a highly branched arabinan in ileal ASS of pigs fed unprocessed RSM, similar in structure to the corresponding water-soluble arabinan. Xyloglucan, originating from RSM, was found next to a branched xylan, of which indications have been found in RSM itself (*4, 5*). Probably this structure is accumulating in unfermented samples. In the fecal ASS obtained from the same pigs, still, branched arabinan, xyloglucan, and galactomannan was present. Next to that, the xylan analyzed in the feces was more linear than in the ileal digesta, indicating that microbial xylan-debranching enzymes were active in the colon. Ileal and fecal samples from pigs fed acid-extruded RSM showed similar glycosidic linkages in the ASS-fractions.

6.3.6.2 Enzymatic fingerprinting of ileal and fecal ASS-fractions

HPSEC-analysis of enzyme digests of the ASS-fraction of feces from pigs fed unprocessed RSM showed activity of arabinanases, galactanases, polygalacturonase, and cellulase towards the ASS-fraction, confirming the presence of arabinan, galactan, homogalacturonan, and cellulose (no further data not shown). In the cellulase-digest also xyloglucan-oligomers were formed, as analyzed by MALDI-TOF MS. These oligomers were XXXG or XSGG, XXLF or LSGG, XXFG and XLFG (Supplemental table 6.2; nomenclature according to Fry et al. (*2*)), indicating the presence of mainly XXXG-type xyloglucan with arabinosyl, galactosyl, and fucosyl residues, as was also indicated for *B. napus* meal (*4*). All NSP-structures present in the various ASS-fractions analyzed are summarized in Supplemental table 6.3.

6.3.6.3 Unextractable carbohydrates in RES

The unextractable residual fraction (RES) mainly contained glucosyl (48-56 mol%) and uronyl residues (22-31 mol%; Table 6.4). Polymeric structures in the RES-fraction could not be accurately analyzed using glycosidic linkage type analysis due to their insolubility in DMSO. Also using enzymatic fingerprinting carbohydrate structures could not be elucidated, since the carbohydrates were inaccessible for the enzymes used to be degraded. It is hypothesized that the RES-fractions mainly contain cellulose and tightly bound pectins (*32, 33*).

In conclusion, ileal NSP-fermentability of RSM in pigs was 22%, and fecal fermentability was 68%. Acid-extrusion could numerically slightly improve the total tract NSP-fermentability to 72%. Nevertheless, acid-extrusion of RSM decreased the amount of water-soluble and increased the amount of residual carbohydrates in ileal digesta. Probably, acid-extrusion tightened this ileal NSP-matrix. Disappearance of constituent monosaccharides occurred gradually and evenly when the digesta moved towards the distal part of the digestive tract.

During alkaline extraction using 6M NaOH, 35-54% (w/w) of the unfermented water unextractable carbohydrates from digesta and fecal samples were unexpectedly released as small uronyl-rich carbohydrates (<14 kDa). It follows that alkali-labile bonds hinder the complete fermentation of NSP in pigs.

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Supplemental data

Supplemental table 6.1. Content, yield, and recovery of dry matter and protein from water and alkaline extraction of ileal and fecal samples from pigs fed a diet containing unprocessed RSM (Unpr. RSM) and acid-extruded RSM (Acid RSM).

	Dry matter	Protein	
	Yield (%)	Content (% w/w)	Yield (%)
Unpr. RSM			
Ileum	100	19	100
WSS	*54*	26	*72*
ASS	*10*	4	*2*
RES	*16*	8	*7*
Recovery (%)	***81***		***81***
Feces	100	23	100
WSS	*19*	21	*17*
ASS	*8*	27	*9*
RES	*42*	11	*20*
Recovery (%)	***69***		***46***
Acid RSM			
Ileum	100	15	100
WSS	*38*	20	*50*
ASS	*18*	5	*6*
RES	*26*	8	*14*
Recovery (%)	***82***		***70***
Feces	100	23	100
WSS	*19*	23	*20*
ASS	*11*	30	*14*
RES	*43*	12	*23*
Recovery (%)	***73***		***57***

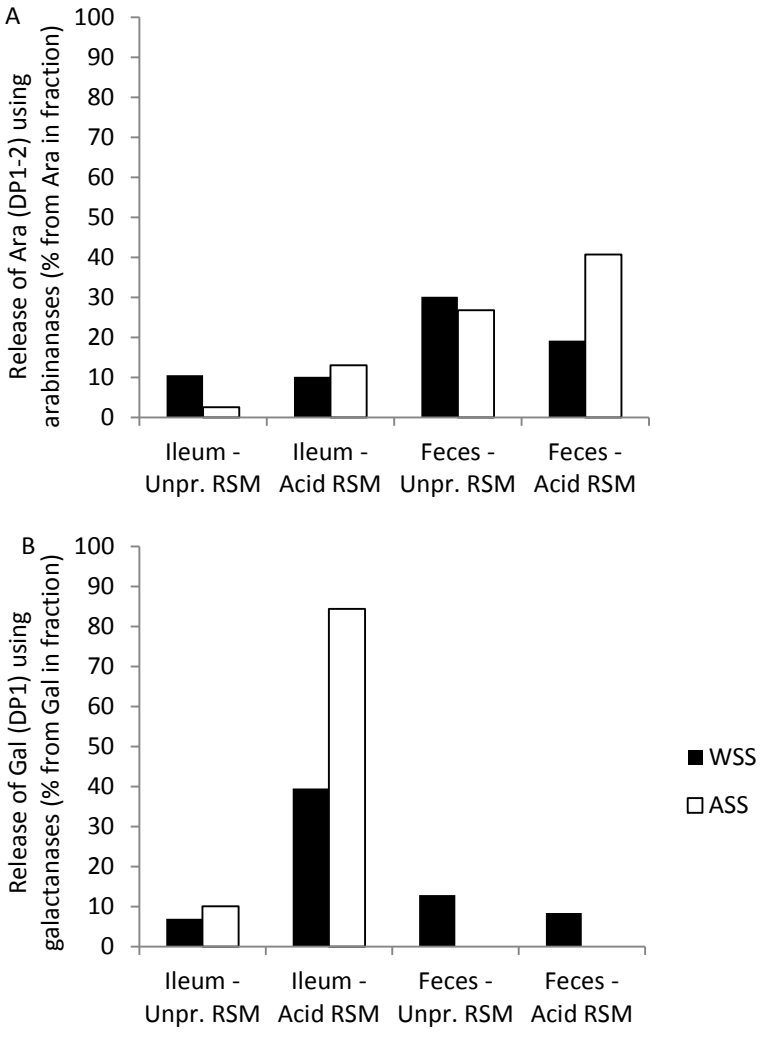
Supplemental table 6.2. Presence of oligosaccharide fragments after enzymatic fingerprinting of the alkali-solubles >10 kDa (ASS) and final residue (RES) of ileal digesta and feces after in vivo digestion in pigs fed diets containing unprocessed (Unpr. RSM) and acid-extruded (Acid RSM) RSM. (Hex=hexose, Pen= pentose, UA= uronic acid, OMe= 4-O-methylgroup).

	Unpr. RSM				Acid-RSM			
	Ileum		Feces		Ileum		Feces	
Structure	ASS	RES	ASS	RES	ASS	RES	ASS	RES
Using arabinanases:								
$(Pen)_n$ (max. n) [a]	+ (14)	-	+ (6)	-	+ (14)	+ (7)	+ (6)	-
551 [b]+$(Pen)_n$ (max. n)	+ (10)	-	-	-	+ (10)	-	-	-
Using xylanases:								
$(Pen)_n$ (max. n)	+ (14)	-	+ (6)	-	+ (14)	+ (7)	+ (6)	-
$(Pen)_n$-UA-OMe (max. n)	+ (8)	-	+ (8)	-	+ (10)	-	+ (8)	-
Using XG-specific glucanase:								
Xyloglucan	+	+	+	+	+	+	+	-
Using cellulase:								
$(Hex)_n$ (max. n)	-	-	+ (6)	-	+ (12)	-	+ (9)	-
629 [b]+$(Hex)_n$ (max. n)	+ (3)	-	+ (3)	-	+ (3)	-	+ (3)	-
XXXG/XSGG, XXLG/LSGG, XXFG, XLFG [c]	+	+	+	+	+	+	+	-

[a] oligosaccharides of $DP_{n.}$ [b] As analyzed using MALDI-TOF MS. [c] nomenclature of xyloglucan-oligomers according to Fry et al. (*2*)

Supplemental table 6.3. Summary of polysaccharides present in ileal digesta and feces from pigs fed unprocessed RSM (Unpr. RSM) and acid-extruded RSM (Acid RSM).

	Unpr. RSM				Acid RSM			
	Ileum		Feces		Ileum		Feces	
	WSS	ASS	WSS	ASS	WSS	ASS	WSS	ASS
Branched arabinan at either O2 or O3	+	+		+	+	+		+
Branched arabinan at both O2 and O3	+	+	+		+	+	+	
Linear galactan	+		+	+	+		+	+
Linear xylan				+				+
Glucurono-arabinoxylan		+				+		
Xyloglucan	+	+	+	+	+	+	+	+
Homogalacturonan		+		+		+		+
Rhamnogalacturonan			+	+			+	+
Galactomannan			+				+	+
Residual starch	+				+			
Microbial glucans	+		+		+		+	



Supplemental figure 6.1. Release of arabinose (DP 1-2) (A) and galactose (DP1) (B) after addition of endo- and exo-arabinanase, and beta-galactosidase and endo-galactanase, respectively, from the water- (WSS) and alkali-soluble (ASS) of ileal and fecal samples after in vivo digestion in pigs fed diets containing unprocessed (Unpr. RSM) and acid-extruded RSM (Acid RSM).

Chapter 7

General discussion

7.1 Motivation of the research

The research described in this PhD-thesis was part of a larger project entitled "Improved utilization of complex carbohydrates in poultry and pigs by novel technologies". The project aimed at improving the utilization of non-starch polysaccharides (NSP) in animal feed from co-products of the grain ethanol and seed oil industry, in order to reduce feed costs and enhance sustainable use of feed resources.

As part of this project, the presented thesis focused on rapeseed meal (RSM), which is a cheap and increasingly abundant co-product of the rapeseed oil production. The NSP-structures in RSM were characterized, and their fate was assessed in *in vitro* and *in vivo* trials. Solubilization of NSP was studied *in vitro* and fermentation of NSP was studied *in vivo* in broilers and growing pigs. In addition, the effect of processing technologies and the use of enzymes on the NSP-structures and their fermentation was examined.

7.2 Characterization of rapeseed meal polysaccharides

Rapeseed meal is rich in NSP, which cannot be degraded by the animals' own digestive enzymes. In literature, only NSP from RSM of the *Brassica campestris* variety has been characterized (*2*), while the NSP from RSM analyzed in this thesis was *Brassica napus*. Although both varieties belong to the same phylogenetic family of *Brassicaceae*, besides similar polysaccharides (homogalacturonan, arabinogalactan, and glucuronoxylan), also distinct differences in cell wall polysaccharide structures were found (Chapter 2 (*3*)). *B. napus* contained arabinan with only branches at the O2-position, while *B. campestris* showed branching at O2 and O3. In addition, *B. napus* showed XXGG-type xyloglucan, besides XXXG-type xyloglucan, which was not found in *B. campestris*.

The RSM batches used in this thesis were all of the *Brassica napus* variety, but the carbohydrate-contents was rather variable, ranging from 27 to 47% (w/w). The molar proportion of neutral constituent monosaccharides was comparable, while the contents of uronyl residues was more variable.

Besides the observed differences in NSP-structures in different rapeseed varieties, variation in NSP-structures can also be caused by different treatments during industrial oil-extraction. Depending on the severity of heat and shear used during processing of the seed and the type of extraction (pressing, using solvent or using enzymes), NSP-structures might be affected (*4, 5*). The content and composition of NSP was not affected by acid-

extrusion in Chapter 5 (at 1.4% (w/w) maleic acid), while the NSP-content in Chapter 6 (at 2% (w/w) maleic acid) was slightly higher after acid-extrusion compared with unprocessed RSM. This was not expected to be a result from acid-extrusion. It can be stated that taking a homogeneous sample from this large batch is difficult. These differences complicate interpretation of results from different digestion studies. Nevertheless, the structural differences between varieties are expected to be of greater importance than the effect of treatments during oil processing, while the latter may influence the NSP-content more rather than the NSP-structures.

7.3 Processing of rapeseed meal

RSM has a rigid cell wall matrix (as concluded in Chapter 2 (*3*)). Therefore, different processing technologies were explored to improve accessibility of the cell wall polysaccharides for microbial enzymes, thereby increasing their utilization in monogastric animals. The technologies evaluated use shear, heat, and acid, with or without an enzymatic treatment.

7.3.1 Enzymatic treatment

In a preliminary experiment, different (combinations of) commercial enzyme preparations with mainly pectolytic and some hemicellulolytic activities were tested for their solubilization of carbohydrates (Textbox 7.1).

The enzymes tested were chosen for their strong pectolytic effect based on experience in our group (Laboratory of Food Chemistry, Wageningen University).

Textbox 7.1 - Testing (combinations of) commercial enzyme mixtures

Unprocessed RSM was incubated with (combinations of) commercial enzyme mixtures (~5 mg substrate/mL 10mM NaOAc at pH5.0, enzyme dosage 0.25 µL/5 mg substrate) for 24h at 40°C, rotating head-over-tail. Enzymes were inactivated (10 min, 100°C) and solubilization of carbohydrates was analyzed using an automated orcinol-colorimetric assay (*6*). Arabinose was used for calibration.

The enzyme preparations Pectinex UltraSP-L (*Aspergillus aculeatus;* Novozymes, Bagsvaerd, Denmark) and Multifect Pectinase FE (*Aspergillus niger;* Genencor, Rochester, NY, USA) together act on a broad range of polysaccharides, like arabinan, arabinoxylan,

galactan, galactomannan, β-glucan, homogalacturonan, rhamnogalacturonan, and xyloglucan (personal communication, U. R. Ramaswamy, Laboratory of Food Chemistry, Wageningen University). The combination of Pectinex UltraSP-L and Multifect Pectinase FE showed the highest solubilization of carbohydrates from RSM (Table 7.1). Therefore, it was chosen in the *in vitro* study (Chapter 3 (*1*)) and *in vivo* in broilers (Chapters 4 and 5).

Table 7.1. Increased solubilization of carbohydrates from rapeseed meal by (a combination of) different commercial enzyme preparations.

Commercial enzyme preparation	Carbohydrate solubilization (%) [a]
Blank (no enzyme)	100
Pectinex UltraSP-L (Novozymes)	155
Multifect Pectinase FE (Genencore)	165
Rapidase Liq+ (DSM)	160
Pectinex UltraSP + Multifect Pectinase	174

[a] Increase of water-soluble carbohydrates from RSM after addition of enzymes, compared with the concentration of water-soluble carbohydrates from RSM without enzymes added.

For the *in vivo* trial in pigs, commercial pectolytic enzymes were not added to the diet, because the pigs' colon has a higher fermentative capacity compared with that of broilers (*7*). Also, microbiota of pigs have shown to produce enzymes active towards β-glucans, arabinoxylan, and soy soluble polysaccharides (*8*). The longer residence time in the pigs' colon compared with the broilers' colon is expected to facilitate enzyme activity by microbiota in the colon, which leads to higher fermentability of NSP. The purpose of enzyme addition in broilers' diets is to reduce viscosity of the digesta caused by soluble NSP, this is of less importance for pigs.

7.3.2 Scaling-up acid treatment

In the *in vitro* study described in Chapter 3 (*1*), acid treatment of RSM was performed in an autoclave (~50 g), while for *in vivo* experiments (Chapters 5 and 6) the treatment had to be scaled-up to around 200 kg. Therefore, acid-treatment was performed in an extruder, for which different settings were first tested (Textbox 7.2).

After extrusion with 2% (w/w) maleic acid and 40% (w/w) moisture, solubilization of carbohydrates by enzymes was highest (81% (w/w)) (Figure 7.1). Although extrusion and enzyme incubation were only performed once and not all concentrations were tested, the

results may indicate that at around 2% (w/w) maleic acid accessibility of the RSM cell wall structure for enzyme degradation was optimal. Therefore, these settings were chosen for

Textbox 7.2 - Testing extruder settings

RSM was soaked in increasing concentrations of maleic acid (0-5%) at 40% moisture for 1 hour prior to extrusion. To evaluate effectiveness of extrusion, samples (~5 mg substrate/mL 10mM NaOAc buffer pH5.0) were incubated with commercial pectolytic enzymes (Pectinex UltraSP and Multifect Pectinase FE, enzyme dosage for each enzyme 0.125 µL/5 mg substrate) for 24h at 40°C, rotating head-over-tail. Enzymes were inactivated (10 min, 100°C) and solubilization of carbohydrates was analyzed using an automated orcinol-colorimetric assay (*6*). Arabinose was used for calibration. Extrusion temperature and through-put were chosen at the highest settings possible without burning the substrate (as described in Chapters 3, 5 and 6).

the *in vivo* study in pigs (Chapter 6). For the trial with broilers (Chapter 5) the same acid concentration as tested *in vitro* was used.

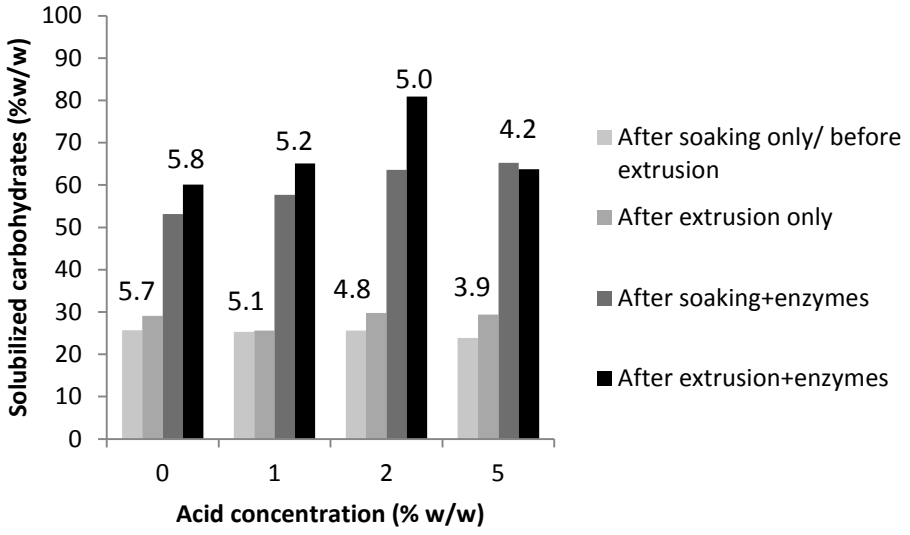


Figure 7.1. Solubilization of carbohydrates of total carbohydrates present (% w/w), after incubation with commercial pectolytic enzymes, before and after extrusion of RSM in the presence of maleic acid. Numbers on top of the bars represent the pH-values of the samples.

7.4 Comparison of the fate of NSP from (processed) RSM in *in vitro* and *in vivo* studies

7.4.1 Fate of total NSP from RSM

The aim of the *in vitro* trial was to evaluate processing of RSM and to predict fermentation of (processed) RSM *in vivo*. *In vitro* this was judged based on solubility of NSP (Boisen incubation; Chapter 3 (*1*)), since solubilized NSP are assumed to be easily fermented *in vivo*. Wet-milling, extrusion, and acid treatment, only when combined with addition of commercial pectolytic enzymes, showed to be able to significantly improve solubilization of NSP from RSM. About 30-35% of the carbohydrates remained in the insoluble residue. Especially solubilization of arabinosyl and uronyl residues was improved (Table 7.2).

Similar to results from the *in vitro* assay, in broilers (Chapter 4) addition of commercial pectolytic enzymes showed to improve fermentability of non-glucose polysaccharides (NGP) significantly, while wet-milling, extrusion or acid-treatment alone could not (Table 7.2). Improvement of fermentability of constituent monosaccharides was mainly seen for arabinosyl and xylosyl residues. Unfermented carbohydrate structures in the broilers' excreta were highly branched (Chapter 5), thereby hindering degradation by enzymes. Furthermore, in broilers the addition of pectolytic enzymes to unprocessed RSM had a significant positive effect on NSP-fermentability, but not when added to acid-extruded RSM. This suggested that acid-extrusion made the NSP-matrix less accessible for the enzymes.

From the results seen *in vitro*, in the pigs' trial (Chapter 6) it was expected that acid-extrusion of RSM could solubilize NSP, which would give an increase in fermentability of acid-extruded RSM compared with unprocessed RSM. However, acid-extrusion of RSM numerically decreased cecal NSP-fermentability and total tract NSP-fermentability increased with only 4% points (Table 7.2). Opposed to our hypotheses, acid-extrusion seemed to increase rigidity of the NSP-matrix rather than to loosen it, in both excreta of broilers and ileal digesta of pigs. In pigs, ileal fermentability of non-starch polysaccharides (22%) from unprocessed RSM was much lower than *in vitro* solubility (50%). Apparently, the predicted increase in NSP-solubilization *in vitro* is not a guarantee for improved NSP-fermentability *in vivo*. This may be due to the fact that little fermentation takes place in the pig's ileum and in the broilers' ceca. Microbial enzymes may be able to degrade NSP

Table 7.2. Comparison of solubilization of NSP and arabinosyl (Ara), xylosyl (Xyl), glucosyl (Glc), and uronyl (UA) residues *in vitro* (Chapter 3) with fermentability *in vivo* in broilers fed unprocessed or acid-extruded RSM with and without the addition of commercial pectolytic enzymes (Chapter 5) and in pigs fed unprocessed (Unpr.) and acid-treated (Acid) RSM (Chapter 6). Solubility/fermentability was calculated as described in Chapter 3 (in vitro (*1*)), 5 (broilers), and 6 (pigs).

	In vitro - Upper GIT				*In vivo* broilers – Total tract				*In vivo* pigs - Ileal		*In vivo* pigs - Total tract	
	Unpr.	Unpr. +[a]	Acid	Acid [a]	Unpr.	Unpr. + [a]	Acid	Acid [a]	Unpr.	Acid	Unpr.	Acid
Total NSP/NGP [b]	50	54	49	69	24	38	29	32	22	23	68	72
Ara	9	30	42	79	22	41	30	38	11	12	85	85
Xyl	22	37	25	67	31	41	37	39	25	3	79	82
Glc [c]	58	49	51	66	na [d]	na	na	na	37	43	74	75
UA	60	66	54	58	31	43	32	34	18	10	51	58

[a] Including the addition of commercial pectolytic enzymes (+). [b] Non-glucose polysaccharide fermentability was calculated for broilers. [c] It should be remarked that a small amount of (partly digested) starch and microbial glucans are included in this value, next to xyloglucan and cellulose from RSM. [d] na= not analyzed.

later in the digestive tract where substantial fermentation of the solubilized NSP is expected (*7*).

7.4.2 Fate of water-soluble polysaccharides in broilers' excreta and pigs' feces

Broilers could not ferment 62-76% of the carbohydrates in the diet, from which 16-18% (w/w) was water-soluble (Chapter 5). Pigs could not ferment 28-32% of the NSP in the diet, from which only 4-5% (w/w) was water-soluble (Chapter 6). Pigs have a higher fermentative capacity than broilers and were capable of almost completely fermenting water-soluble carbohydrates, as was also described in literature (*7*). Unfermented water-soluble carbohydrates remaining in the excreta of broilers and in the feces of pigs fed (processed) RSM were compared (Table 7.3). In excreta of broilers fed unprocessed RSM branched arabinan, linear galactan, homogalacturonan, and (xylo-)glucan were the main water-soluble polysaccharides present, as was determined using linkage type analysis.

Table 7.3. The presence of water-soluble carbohydrate structures in the excreta of broilers (Chapter 5) and feces of pigs (Chapter 6) fed unprocessed (Unpr.) and acid-extruded (Acid) RSM (x indicates that a structure is present; -indicates that the structure is not present).

	In vivo broilers				*In vivo* pigs	
	Unpr.	Unpr. + [a]	Acid	Acid + [a]	Unpr.	Acid
Small oligosaccharides [b]	x	x	x	x	-	-
Linear arabinan	-	x	-	x	x	x
Single substituted arabinan	x	-	x	-	-	-
Double substituted arabinan	-	-	-	-	x	x
Galactan	x	x	x	x	x	x
Xyloglucan	x	x	x	x	x	x
Homogalacturonan	x	x	x	x	x	x
Rhamnogalacturonan	-	-	-	-	x	x
Galactomannan	x	x	x	x	x	x

[a] Including the addition of commercial pectolytic enzymes (+). [b] Small oligosaccharides being fructose, raffinose, and stachyose.

The use of commercial pectolytic enzymes in the broilers' diet decreased branching of the water-soluble arabinan, also reflected in a significant improvement of fermentability of arabinosyl residues (Chapter 5).

Similar to broilers, in growing pigs, arabinan, galactan, xyloglucan, homogalacturonan, and galactomannan, remained in the water-soluble fractions of the feces. Differences between broilers' excreta and pigs' feces are mainly seen in the branchiness of the arabinan and the fact that a water-soluble rhamnogalacturonan was found in pigs' feces. The water-soluble arabinan found in pigs' feces is more linear than in broilers' feces, indicating that microbiota in the pigs colon produced more arabinofuranosidase activity. Rhamnogalacturonan was solubilized by enzyme activity of the microbiota in the pigs' colon, because in the RSM itself it was present in the unextractable residual fraction (Chapter 2 (*3*)). In broilers, the rhamnogalacturonan remained unextractable from the excreta (Chapter 5).

These water-soluble carbohydrate structures in broilers' excreta and ileal digesta and feces from pigs have never been reported before. So, no comparison with literature is possible. In animal nutrition research, the WSS-fraction is not analyzed as part of the neutral detergent fiber (NDF), acid detergent fiber (ADF), or acid detergent lignin (ADL), nor is it the same as ethanol soluble sugars (Section 1.4.1). Our findings give new insights on fermentability of water-soluble carbohydrate structures and effectiveness of processing and enzymes used.

7.4.3 Fate of recovered alkali-soluble polysaccharides in broilers' excreta and pigs' feces

In excreta from broilers fed different diets, 4-17% (w/w) of the carbohydrates was recovered in the alkali-soluble fraction (ASS; Chapter 5), and in pigs' feces this was 7-14% (w/w) (Chapter 6). It should be noted that pigs could ferment more of the NSP than broilers (Table 7.2). Therefore, the absolute amount of NSP recovered in this ASS-fraction was lower for pigs compared with broilers. Unfermented carbohydrates in ASS from excreta from broilers and feces from pigs were compared (Table 7.4). In the ASS-fraction from broilers' excreta mainly (glucurono)xylan, xyloglucan and some galactomannan were still present. Similar to broilers' excreta, in the ASS-fraction from pigs' feces xylan, xyloglucan, and galactomannan were detected. Different from the ASS of broilers' excreta, in pigs' feces also branched arabinan and galactan were found. Also, the alkali-soluble xylan in broilers' feces is more linear than the alkali-soluble xylan in pigs' feces, indicating that microbiota in the pigs' colon produce xylan-debranching enzymes. In the broilers'

diets, besides RSM also maize was included from which NSP-structures in the excreta can also originate. Acid-extrusion resulted in similar NSP-structures in the corresponding fecal ASS-fractions. Again, such structures have never been analyzed before in similar samples and are not covered by NDF, ADF or ADL determinations, as used in animal nutrition research.

Table 7.4. Carbohydrate structures in the ASS-fractions from excreta of broilers (Chapter 5) and feces of pigs (Chapter 6) fed unprocessed (Unpr.) and acid-extruded (Acid) RSM (x indicates that a structure is present; -indicates that the structure is not present).

	In vivo broilers				*In vivo* pigs	
	Unpr.	Unpr. + [a]	Acid	Acid + [a]	Unpr.	Acid
Single substituted arabinan	-	-	-	-	x	x
Galactan	-	-	-	-	x	x
Arabinogalactan type II	-	-	-	-	-	-
Linear xylan	x	x	x	x	x	x
Glucuronoxylan	x	x	x	x	-	-
XXGG-type xyloglucan	x	x	x	x	x	x
XXXG-type xyloglucan	x	x	x	x	x	x
Rhamnogalacturonan	-	-	-	-	-	-
Galactomannan	x	x	x	x	x	x

[a] Including the addition of commercial pectolytic enzymes (+).

7.4.4 Fate of other (calculated) alkali-soluble carbohydrates

During alkaline extraction using 6M NaOH, 39-54% of the water unextractable carbohydrates of excreta from broilers and feces from pigs were, unexpectedly, not recovered in the ASS- or RES-fractions (Chapters 5 and 6). The amount and molar composition of this unrecovered fraction was calculated, providing some indications about their structures, but the exact nature of these carbohydrates remained unclear. Nevertheless, in an attempt to know more about these carbohydrate structures, extraction and dialysis were repeated on a smaller scale (Textbox 7.3).

The HPSEC-chromatograms of ASS from excreta from broilers and feces from pigs, showed elution of material around 270 kDa for broilers and 32 kDa for pigs (results not shown). From HPSEC-analysis of the alkali-soluble carbohydrates retained in the dialysis water (ASSd), results were inconclusive due to the presence of high amounts of salts. Still, ASSd showed a reasonable detection on HPAEC and a variety of peaks was shown for ASSd of excreta from broilers (Figures 7.2A and 7.2B) and feces from pigs (Figures 7.2C and 7.2D).

Because in HPAEC, combined with PAD-detection, peaks represent elution of carbohydrates, it can be concluded that in ASSd a variety of different oligosaccharides was recovered and that indeed oligosaccharides < 14 kDa are released during alkaline extraction.

Textbox 7.3 - Alkali extraction on small scale

Water unextractable solids of excreta from broilers and feces from pigs fed a diet containing unprocessed RSM and acid-extruded RSM (~500 mg), and a fecal sample from pigs fed a diet without RSM (samples from a study by Haenen and Souza Da Silva, Division of Human Nutrition and Animal Nutrition Group, Wageningen University, to be published) were extracted using 20mL 6M NaOH containing 20mM $NaBH_4$ at 4 °C for twice 1 h (Alkali soluble solids, ASS) rotating head-over-tail. ASS were separated from the insoluble residue, neutralized, dialyzed in distilled water at 4°C overnight and freeze-dried. Also, the dialysis water was freeze-dried (ASSd). ASS and ASSd were analyzed by HPSEC and ASSd was analyzed by HPAEC (both methods described in Chapters 5 and 6). Similarly, dialysis was also performed for a standard dextran. From this, 95% could be recovered, indicating that a good yield can be achieved with the dialysis tubings used.

As discussed in Chapters 5 and 6, we can hypothesize three explanations on the origin of these carbohydrates in ASSd. First, these carbohydrates were ester-linked in the WUS-fractions in ileal digesta, excreta and feces, to e.g. lignin (*9*). Second, 6M NaOH helped cellulose fibrils to swell, as published for bamboo fibers (*10*), thereby carbohydrate-structures entrapped by H-bonding in WUS-fractions were released by alkali. Third, small carbohydrates originating from RSM NSP could be bound to cells of microbiota mediated by a receptor, present in the colon, as part of their digestive mechanism (*11*). To test relevance of this third option, water unextractable solids of feces from pigs fed a diet without RSM (instead soy, sugar beet, wheat, and barley were present as NSP-sources in the diet) was also extracted using 6M NaOH (as described in Textbox 7.4). From this sample, 26% (w/w) of the water unextractable carbohydrates were retained in the ASS and 71% (w/w) was found in the final residue. Hence, carbohydrate recovery of this extraction was 97%, leaving only 3% (w/w) of the carbohydrates in the calculated ASSd-fraction. This implied that the alkali-labile linkages within the NSP-matrix or to the microbial cell are specific for RSM. Also, a similar extraction of NSP from RSM added to the diets did not result in such a release of small carbohydrates.

In broilers' excreta these unfermented carbohydrates in the ASSd-fraction were calculated to be rich in glucosyl and uronyl residues (Chapter 5) and from pigs' feces these carbohydrates were rich in uronyl residues (Chapter 6). These carbohydrates could originate from pectic polysaccharides, which are ester-linked to phenolic acids in insoluble material. For sugar beet and spinach, feruloylation of pectins has been described at the O2-position of α-1,5-linked-arabinofuranosyl residues in the arabinan side chains (*12*) and at the O6-position of β-1,4-linked galactosyl residues (*13*). In theory, also ester-linkages via the acid-group of galacturonic acid can be present (*14*). Elucidation of the origin of these structures is an important item for continuation of this research.

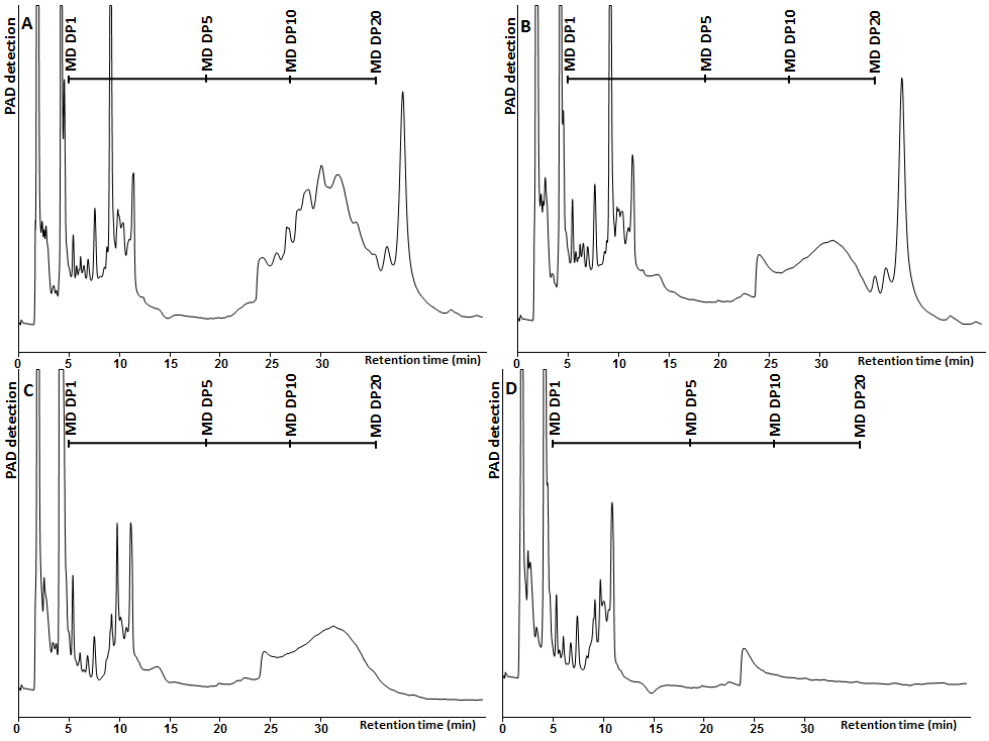


Figure 7.2. HPAEC chromatograms of ASSd (dialysate) of excreta from broilers fed unprocessed RSM (A) and broilers fed acid-extruded RSM (B), and feces from pigs fed unprocessed RSM (C) and pigs fed acid-extruded RSM (D). MD= Maltodextrin; DP= Degree of Polymerization.

The carbohydrates in ASSd (dialysate) could also originate from homogalacturonan cross-linked to cellulose by hydrogen bridges and hydrophobic interactions (*15*) or xyloglucan bound to cellulose by H-bonding (*16*). The finding of such alkali-labile NSP-linkages remaining in excreta of broilers and digesta and feces of pigs would not have been analyzed in the NDF, ADF, and ADL-methodology used.

7.4.5 Fate of unextractable carbohydrates

After water and alkaline extraction still around 40% of the carbohydrates in broilers' excreta and pigs' feces remained in the residues (Chapters 5 and 6). Polymeric structures were difficult to analyze, but constituent monosaccharide composition indicated the presence of cellulose and tightly bound pectins (e.g. rhamnogalacturonan and arabinan) and xyloglucan, as described in literature (*17, 18*). This residual fraction corresponded partly with the ADF, but still other carbohydrates remain unextractable besides cellulose.

7.4.6 Microbiota in pigs' colon

Carbohydrate metabolism is known to correspond with the most abundant microbial genes in pig microbiota (*19*). Any difference in carbohydrate utilization is expected to provoke changes in the microbiota composition. In an additional experiment, complementary to our fermentation study (Chapter 6), microbial composition in the pigs' colon was analyzed using a phylogenetic microarray, targeting pigs' intestinal tract microbiota (Pig Intestinal Tract Chip, PIT-chip) (Textbox 7.4).

Textbox 7.4 - Microbiota composition in pig colon samples

Digesta from proximal, mid, and distal colon (Chapter 6) were analyzed for their microbiota composition using the PIT-chip as described elsewhere (*20*).

The relative abundance of the most abundant (with relative abundance <1%) groups of species is shown in Figure 7.3. In the proximal colon of pigs fed unprocessed RSM, the most abundant species were *Bacilli* (27%), *Clostridium* cluster IV (28%), and *Clostridium* cluster XIVa (19%). These three groups belong to the phylum *Firmicutes*, which together with *Bacteroidetes* form the two most abundant phyla in microbiota in the pig's colon (*21*).

The relative abundance of *Bacteroidetes* in the proximal colon was rather low (3%), while it was more abundant in mid and distal colon (13% and 19%, respectively). The relative abundance of *Bacilli* seems to decrease from proximal to mid colon and *Clostridium* cluster IV seems to decrease from mid to distal colon. Other microbiota groups were relatively stable.

The relative abundance of the species mentioned in Figure 7.3 is rather similar to the microbiota composition in the pigs' intestine as published before (*22*), except that the relative abundance of Clostridium cluster IV was somewhat higher and Clostridium cluster XIVa somewhat lower.

Although the experimental set-up did not allow statistical analysis, the results indicated that acid-extrusion of RSM in the diet tends to decrease relative abundance of *Clostridium* cluster XVI in proximal colon. Also, relative abundance of *Bacilli* seems to be decreased in mid and distal colon after acid-extrusion. On the other hand, relative abundance of *Bacteroidetes* seems to be increased in proximal and mid colon upon acid-extrusion. It is still unknown how these microbial compositional changes due to diet relate to digestive functions and animal wellbeing (*21*).

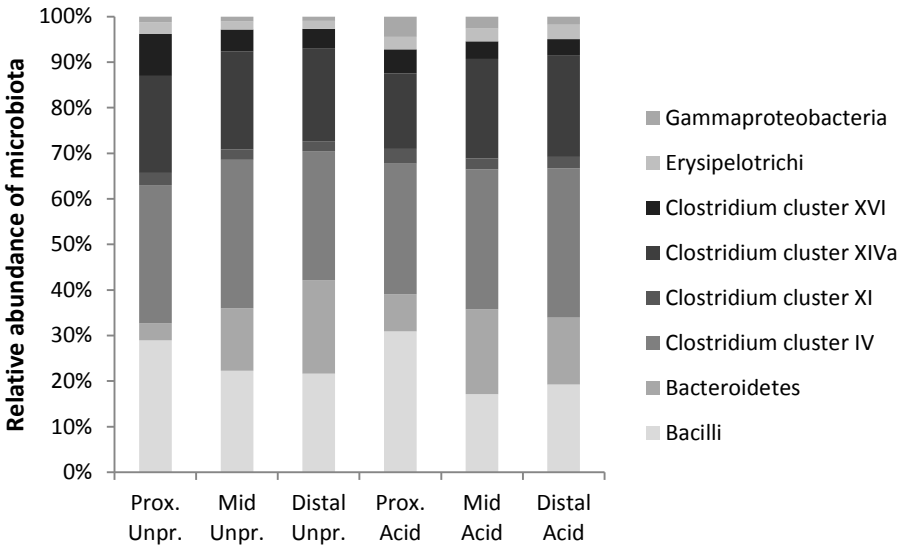


Figure 7.3. Relative abundance (<1%) of microbiota in samples taken in different parts of the colon from pigs fed a diet containing unprocessed or acid-extruded RSM (Chapter 6).

When comparing the most abundant microbiota species, the composition in the pigs' intestine seems to be rather similar to that in the chicken ceca in terms of *Clostridium* cluster IV, *Clostridium* cluster XIVa and other Gram-positive bacteria (*22*). Only Bacteroides seem to be more abundant in pigs' intestine than in the chickens' ceca. The similarity between microbiota composition in chicken and pigs suggests that the chicken microbiota would also be able to degrade more carbohydrate structures similar to pigs, but that the residence time is the limiting factor in NSP-fermentation in chicken.

Cluster analysis of different samples (Figure 7.4) showed that the microbiota in proximal colon of pigs fed unprocessed and acid-extruded RSM were relatively similar, while those of the mid and distal colon seem to deviate more. This means that NSP influenced by acid-extrusion of RSM in the diet has more effect on microbiota in the mid and distal colon than on the proximal part of the pigs' colon. This coincided with a rather similar total NSP-fermentability in the proximal colon (67-69%), but numerically improved NSP-fermentability in the distal colon (70% in pigs fed unprocessed RSM versus 76% in pigs fed acid-extruded RSM). Apparently, the microbiota present in the mid and distal part of the colon produced enzymes to make this difference in NSP-fermentation.

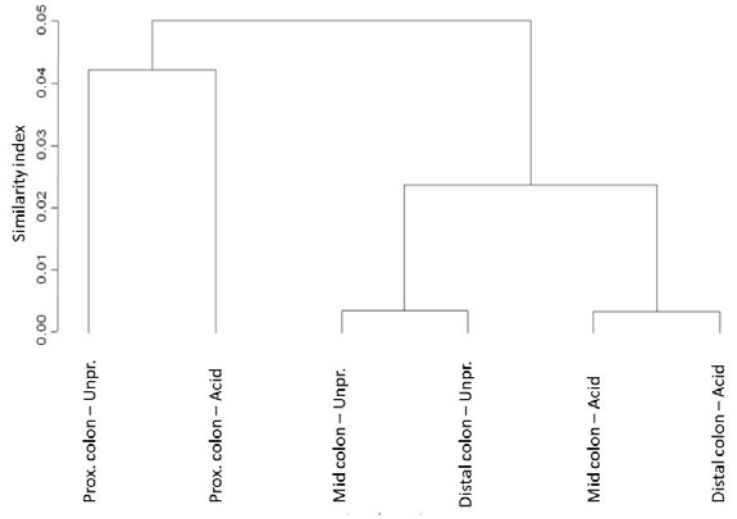


Figure 7.4. Cluster analysis of microbiota in samples taken in different parts of the pigs' colon from pigs fed a diet containing unprocessed (Unpr.) or acid-extruded (Acid) RSM (Chapter 6).

7.5 Future perspectives

7.5.1 Processing technologies and enzyme addition to further increase NSP fermentability of RSM

In Table 7.5 the carbohydrate structures in unprocessed RSM (Chapter 2) and in excreta of broilers (Chapter 5) and feces of pigs (Chapter 6) fed unprocessed and acid-extruded RSM is summarized. Broilers were able to ferment single substituted arabinan and arabinogalactan type II. Microbiota in pigs were able to ferment small saccharides (sucrose, raffinose, stachyose), arabinogalactan, and were able to debranch glucurono-arabinoxylan. All other carbohydrate structures present in RSM were still present, indicating they could only be partly fermented by the animals.

Further improvement of NSP-fermentability in broilers is expected when additional enzyme activities are selected. Unfermented carbohydrate structures in the broilers' excreta were highly branched (Chapter 5), thereby hindering degradation by microbial enzymes. Specifically, the addition of debranching and exo-acting enzymes towards branched arabinan, arabinoxylan, galactomannan, or xyloglucan is suggested. Also, a commercial cellulase mixture is expected to be a good candidate for further improvement, since it helps to degrade cellulose (and other β-glucans). In addition, aided by cellulase addition, NSP strongly attached to the cellulose fibrils can be released, such as xyloglucan (*16*), arabinan (*23*), and rhamnogalacturonan (*17*). Still, it is not known whether the residence time in the broilers' digestive tract is long enough for the enzymes to degrade and solubilize NSP.

For pigs, it is concluded that most water-soluble carbohydrates can be fermented by the microbiota present. Therefore, technologies should aim at specific solubilization of carbohydrate structures that cannot be solubilized by microbial enzymes in the pigs' colon. To achieve this, more severe conditions could be tested, e.g. a stronger acid or a longer residence time during processing.

In both broilers and pigs, unfermented carbohydrates released upon alkaline conditions, indicating that the nature of these carbohydrates (e.g. interactions via ester-linkages or H-bonding) limit their utilization in RSM specifically. Therefore, alkaline treatment could be useful to break these RSM-linkages and to improve NSP-fermentability in pigs and poultry. Already, alkaline extrusion of rapeseed meal has been shown to improve nutritional value in chicks (*24*) and alkaline peroxide treatment of *B. campestris* straw improved nutrient

Table 7.5. Summary of the carbohydrate structures present in unprocessed RSM (Chapter 2) and in excreta of broilers (Chapter 5) and feces of pigs (Chapter 6) fed unprocessed (Unpr.) and acid-extruded (Acid) RSM (x indicates that a structure is present; -indicates that the structure is not present).

	Undigested RSM	*In vivo* broilers				*In vivo* pigs	
	Unpr.	Unpr.	Unpr. + [a]	Acid	Acid + [a]	Unpr.	Acid
Small saccharides [b]	x	x	x	x	x	-	-
Single substituted arabinan	x	-	-	-	-	x	x
Double substituted arabinan	-	-	-	-	-	x	x
Galactan	-	-	-	-	-	x	x
Arabinogalactan type II	x	-	-	-	-	-	-
Linear xylan	-	x	x	x	x	x	x
Glucuronoxylan	x	x	x	x	x	-	-
XXGG-type xyloglucan	x	x	x	x	x	x	x
XXXG-type xyloglucan	x	x	x	x	x	x	x
Homogalacturonan	x	x	x	x	x	x	x
Rhamnogalacturonan	x	(x)	(x)	(x)	(x)	x	x
Galactomannan	x	x	x	x	x	x	x
Cellulose	x	x	x	x	x	x	x
UA-rich carbohydrates (<14 kDa)	-	-	-	-	-	x	x
Glc- and UA-rich carbohydrates (<14 kDa)	-	x	x	x	x	-	-

[a] Including the addition of commercial pectolytic enzymes (+). [b] Small saccharides being sucrose, raffinose, and stachyose

utilization in sheep (*25*). Furthermore, treatment of RSM with ammonia is reported to decrease glucosinolate levels (*26*), promotes breakdown of sinapine (*27*), and to increase nitrogen-digestibility from full-fat rapeseed in pigs and chicken (*28*). Nevertheless, the use of alkali also has negative effects, such as the high amount of salts that is needed to neutralize the pH before the pretreated material can be added in the feed. Also, the viscosity of the material resulting from alkaline treatment is rather high. These positive and negative aspects need to be studied further for alkali types and concentrations possibly combined with thermal treatment, like extrusion. If carbohydrates in excreta and feces are really ester-linked in the matrix, another milder and more environmentally friendly option is to use enzymes with esterase-activity. Many types of esterases exist, so investigation of the type of ester-linkage is needed in order to find enzymes with suitable esterase-activity.

7.5.2 Analytical techniques to study NSP in digesta and feces/excreta

Our work provided new insights in the fate of unfermented carbohydrate structures *in vitro* and *in vivo* in broilers and pigs. After extraction of these samples, water- and alkali-soluble carbohydrates could be analyzed in detail using glycosidic linkage type analysis and enzymatic fingerprinting. Unfortunately, two rather important fractions could not be analyzed within the timeframe of this project or by the methods available in our lab. First, the alkali-soluble carbohydrates not retained in the dialysis membrane could only be calculated so far. The nature of the alkali-labile bonds could be studied further. For example, to find evidence for hydrogen bonding, NaOH/urea aqueous solutions could be used for the dissolution of cellulose from water unextractable solids (*29*), thereby releasing carbohydrates linked via hydrogen bonding. To indicate the presence of specific ester-bonds through phenolic acids, WUS-samples could be saponified to release bound phenolic acids, which can be analyzed using UHPLC (*30*). Second, from the unextractable part of the excreta, digesta, and feces, only the constituent monosaccharide composition could be analyzed. Further analysis of this highly insoluble material, could be done by pyrolysis followed by GC-MS identification of the decomposition products from e.g. cellulose, hemicellulose, and lignin (*31*).

The current analysis of ADF and NDF in animal nutrition research, provides data on fermentability of total NSP, but these methods miss important structural aspects of the water-soluble carbohydrates and of specific linkages present, which influence

fermentation. So, to evaluate and compare animal performance on various diets current methodology is sufficient. However, for evaluation of the effects of processing and enzyme addition, the analyses performed in this thesis will provide more details and insights on the effectiveness *in vivo*.

7.6 Concluding remarks

In conclusion, this thesis showed that addition of commercial pectolytic enzymes can significantly improve fermentability of NSP from RSM in broilers. Based on the knowledge obtained on the unfermented carbohydrate structures, additional enzyme activities, like arabinofuranosidases and cellulases, are expected to further improve NSP-fermentability. Processing technologies tested were not able to improve fermentability in broilers significantly. In pigs, acid-extrusion slightly improved NSP-fermentability numerically. Most water-soluble carbohydrates were fermented. So, future technologies should aim at specifically increase solubilization of NSP-structures that cannot be solubilized by microbial enzymes. In both broilers and pigs, ester-linkages or H-bonding seemed to retain up to half of the unfermented carbohydrates, thereby limiting utilization of uronyl- and/or glucosyl-rich NSP. Therefore, addition of esterases or alkaline treatment could be an option to improve NSP-fermentability in future for broilers and pigs.

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Summary

Summary

Rapeseed meal (RSM) is an abundant co-product from rapeseed oil production, which is used in animal diets for its high protein content. Apart from protein, RSM is rich in non-starch polysaccharides (NSP). Animals lack the digestive enzymes to degrade these NSP. Therefore, mechanical, thermal, thermo-mechanical, and chemical technologies, with or without the addition of enzymes, were explored to improve NSP-fermentation from RSM. A range of NSP-structures described in literature and expected to be present in RSM, including their architecture in the plant cell wall were introduced in **Chapter 1**, followed by an explanation of the digestive tract of poultry and pigs, and current methods for processing of raw materials in the animal feed industry.

In **Chapter 2**, the cell wall polysaccharides from RSM were characterized. Pectic polymers, like arabinan, arabinogalactan type II, rhamnogalacturonan and homogalacturonan were analyzed. Also cellulose and hemicelluloses, like XXGG- and XXXG-type xyloglucan, and glucuronoxylan were present. RSM has a rigid matrix, which could not be completely extracted or degraded by enzymes. The carbohydrate structures found in *Brassica napus* meal were compared with those of *Brassica campestris* meal, since the latter meal has been described before in literature. *B. napus* contained arabinan with only O2 branches (instead of branching at O2 and O3) and XXGG-type xyloglucan (besides XXXG-type xyloglucan), which were not found in *B. campestris.* Homogalacturonan, arabinogalactan, and glucuronoxylan were similar in structure.

Several processing technologies, involving shear, heat, and acid, with and without the addition of commercial pectolytic enzymes, were applied to RSM to open up the cell wall matrix. Unprocessed and processed RSM were degraded *in vitro* in **Chapter 3**. From the unprocessed RSM 50% of the NSP remained insoluble. Addition of commercial pectolytic enzymes, only after processing, was able to decrease the amount of residual insoluble carbohydrates significantly. Acid treatment combined with addition of pectolytic enzymes was found to decrease insoluble carbohydrates the most. In this case 31% of the NSP from the initial material remained insoluble. Therefore, this treatment was assumed to be the best option to improve NSP-utilization *in vivo*. This process also significantly decreased the water binding capacity and viscosity. Fermentation of the insoluble *in vitro* degraded carbohydrates was not influenced by the technologies used.

The most promising treatments from the *in vitro* study were tested *in vivo* in broilers for their NSP-fermentability (**Chapter 4**), where 24% of the non-glucose polysaccharides (NGP) from unprocessed RSM (and maize) could be fermented. Addition of commercial

pectolytic enzymes significantly improved NGP-degradation to 38%, while wet milling, extrusion or acid-extrusion alone could not. From this study excreta were selected from broilers fed unprocessed RSM and acid-extruded RSM, with and without the addition of commercial pectolytic enzymes. Unfermented carbohydrate structures in broilers' excreta were characterized in detail in **Chapter 5**. Broilers could not ferment highly branched carbohydrate structures. Mainly XXXG-type xyloglucan, (glucurono-)xylan, (branched) arabinan, and cellulose remained in the excreta. Addition of commercial pectolytic enzymes decreased branchiness of the water-soluble arabinan, thereby significantly improving NSP-utilization. Acid-extrusion alone could not significantly improve NSP-utilization, it even seemed to increase rigidity of the NSP-matrix *in vivo.*

RSM was also fed to pigs (**Chapter 6**) and NSP-fermentation was followed along the digestive tract. In the ileum, the NSP-fermentability was low (22%), but at the end of the digestive tract up to 68% of the NSP could be fermented. The different constituent monosaccharides seem to be gradually fermented between terminal ileum and terminal colon, with nearly all water-soluble carbohydrates fermented at the end of the digestive tract. Mainly rhamnogalacturonan, (branched) arabinan, XXXG-type xyloglucan, linear xylan, galactomannan, and cellulose remained in the feces. Although the experimental set-up did not allow statistical analysis, acid-extrusion of RSM numerically improved NSP-fermentability to 72%. Acid-extrusion decreased branchiness of the water-soluble arabinan, solubilized xyloglucan in ileal digesta, possibly releasing it from its network with cellulose, but it could still not be completely degraded. Acid-extrusion seemed to increase rigidity of the NSP-matrix *in vivo*.

In **Chapter 7**, the findings of the different studies were summarized and discussed. The predictability of *in vivo* fermentation of RSM NSP by the *in vitro* assay was evaluated and unfermented carbohydrate structures in broilers' and pigs' feces were compared. An important finding was that during alkaline extraction of the broilers' excreta and pigs' feces, around 40% (w/w) of the insoluble carbohydrates was released as glucosyl- and/or uronyl-rich carbohydrates, probably originally present via ester-linkages or hydrogen-bonding within the cellulose-lignin network. These linkages are expected to hinder complete NSP-fermentation. Therefore, it was concluded that further research should focus on exploring the use of esterases or alkaline treatment to improve NSP-utilization of RSM in poultry and pigs.

Samenvatting

Raapzaadschroot is een bijproduct van het olie-winningsproces uit raapzaad. Het is ruimschoots beschikbaar en wordt toegevoegd aan diervoeding vanwege het hoge eiwitgehalte. Naast eiwit bevat raapzaadschroot ook veel niet-zetmeel polysachariden (NSP). Net als andere zoogdieren, missen kippen en varkens de verteringsenzymen om deze polysachariden af te breken, maar met name in de dikke darm kunnen deze NSP wel gefermenteerd worden en bijdragen aan de energiehuishouding van de dieren. In dit proefschrift wordt onderzocht of mechanische, thermische, thermo-mechanische en chemische technologieën, al dan niet gecombineerd met toevoeging van enzymen, de fermentatie van NSP uit raapzaadschroot verbeteren. Een samenvatting van de NSP-structuren en de architectuur van de plantencelwand is gegeven in **Hoofdstuk 1**. Daarnaast wordt het maag-darmkanaal van kippen en varkens besproken en huidige methoden voor het bewerken van grondstoffen in de diervoedingsindustrie.

Hoofdstuk 2 beschrijft welke celwandpolysachariden in raapzaadschroot aanwezig zijn. Pectine-achtige polysachariden, zoals arabinaan, arabinogalactaan type II, rhamnogalacturonaan en homogalacturonaan zijn aangetoond. Ook cellulose en hemicelluloses, zoals XXGG- en XXXG-type xyloglucaan en glucuronoxylaan zijn aanwezig. Raapzaadschroot heeft een stevige matrix die niet geheel kan worden geëxtraheerd of door enzymen kan worden afgebroken. De koolhydraatstructuren in *Brassica napus* schroot zijn vergeleken met de structuren in *Brassica campestris* schroot, omdat alleen de laatstgenoemde eerder in de literatuur beschreven zijn. *B. napus* bevat arabinaan die alleen aan de O2-positie vertakt is, in plaats van aan O2- en O3-posities, zoals in *B. campestris*, en XXGG-type xyloglucaan, naast XXXG-type xyloglucaan. Homogalacturonaan, arabinogalactaan en glucuronoxylaan hebben een vergelijkbare structuur in beide *Brassica* soorten.

Verschillende procestechnologieën, gebruik makend van (een combinatie van) shear, warmte en zuur, met en zonder toevoeging van commerciële pectolytische enzymen, zijn toegepast om de celwandmatrix van raapzaadschroot te openen. **Hoofdstuk 3** beschrijft hoe onbehandeld en behandeld raapzaadschroot *in vitro* wordt afgebroken. Van onbehandeld raapzaadschroot blijft 50% van de NSP onoplosbaar. Het toevoegen van commerciële pectolytische enzymen zorgt slechts na technologische behandeling voor een significante verlaging van de hoeveelheid onoplosbare koolhydraten. Met name de zuurbehandeling, gecombineerd met de toevoeging van enzymen is in staat de hoeveelheid onoplosbare koolhydraten te verlagen. In dit geval blijft 31% van de NSP

onoplosbaar. Daarom is deze behandeling gekozen om de benutting van NSP ook *in vivo* te bestuderen. Deze keuze wordt ondersteund door de observatie dat deze behandeling *in vitro* ook het waterbindend vermogen en de viscositeit significant verlaagt. Fermentatie van de onoplosbare *in vitro* afgebroken koolhydraten wordt niet beïnvloed door de gebruikte technologieën.

Hoofdstuk 4 beschrijft het effect op fermenteerbaarheid *in vivo* in vleeskuikens van de meest veelbelovende behandelingen op raapzaadschroot uit de *in vitro* studie. Van de niet-glucose polysachariden (NGP) uit onbehandeld raapzaadschroot (en maïs) kan 24% worden gefermenteerd. De toevoeging van commerciële pectolytische enzymen aan het voer verhoogt de afbraak van NGP naar 38%, terwijl alleen nat malen, extrusie en zuur-extrusie van raapzaadschroot dat niet kunnen. Excreta monsters van vleeskuikens die onbehandeld en met zuur geëxtrudeerde raapzaadschroot gevoerd kregen, al dan niet met toegevoegde enzymen, zijn geselecteerd en aanwezige niet gefermenteerde koolhydraatstructuren zijn in detail gekarakteriseerd (**Hoofdstuk 5**). Vleeskuikens kunnen hoog-vertakte structuren niet fermenteren. Met name XXXG-type xyloglucaan, (glucurono-)xylaan, (vertakte) arabinaan en cellulose blijven in de excreta achter. De toevoeging van commerciële enzymen verlaagt de vertakkingsgraad van de water-oplosbare arabinaan, wat de benutting van deze NSP significant verbetert. Extrusie onder zure condities alleen kan de benutting niet verbeteren. De opbrengst van NSP-extractie van deze excreta blijkt zelfs het laagst, wat suggereert dat zuur-extrusie de NSP-matrix *in vivo* verstevigt.

Raapzaadschroot is ook gevoerd aan varkens en **Hoofdstuk 6** beschrijft de NSP-fermentatie in het maag-darmkanaal. In de dunne darm vindt weinig fermentatie plaats (22%), maar aan het einde van het maag-darmkanaal blijkt 68% van de NSP gefermenteerd te zijn. De verschillende monosacchariden waaruit de polysachariden zijn opgebouwd lijken geleidelijk gefermenteerd te worden in de dikke darm. Met name rhamnogalacturonaan, (vertakte) arabinaan, XXXG-type xyloglucaan, lineaire xylaan, galactomannan en cellulose kunnen niet worden gefermenteerd en blijven achter in de feces. Ook al is vanwege de experimentele set-up statistische evaluatie niet mogelijk, het met zuur extruderen van raapzaadschroot verhoogt de fermenteerbaarheid van de NSP numeriek naar 72%. Zuur-extrusie, in combinatie met de fermentatie-enzymen in het maag-darmkanaal van varkens, resulteert in een verlaging van de vertakkingsgraad van de

water-oplosbare arabinaan en een verhoging van de oplosbaarheid van xyloglucaan in de dunne darm. Beide structuren worden niet volledig gefermenteerd.

In **Hoofdstuk 7** zijn de bevindingen van de verschillende studies samengevat en bediscussieerd. De voorspelbaarheid van *in vivo* fermentaties van NSP uit raapzaadschroot aan de hand van de *in vitro* studie wordt geëvalueerd. Verder worden NSP-structuren die niet gefermenteerd kunnen worden in vleeskuikens en varkens vergeleken. Een belangrijke conclusie is dat tijdens alkalische extractie van zowel de excreta van vleeskuikens als de feces van varkens, ongeveer 40% (w/w) van de onoplosbare koolhydraten in oplossing komen als glucosyl- en/of uronyl-rijke koolhydraten, welke tijdens dialyse door het dialyse-membraan heen diffunderen. Waarschijnlijk zijn deze oorspronkelijk aanwezig via ester-bindingen of waterstofbruggen in het cellulose-lignine netwerk. Blijkbaar hinderen deze bindingen volledige fermentatie van de NSP. Daarom zou verder onderzoek zich moeten richten op het gebruik van esterases of alkalische behandelingen van raapzaadschroot om de benutting van de NSP in kippen en varkens verder te verbeteren.

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Annemieke

About the author

Curriculum vitae

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Curriculum Vitae

Anne Maria (Annemieke) Pustjens was born on the 3rd of October 1984 in Maastricht, The Netherlands. After graduating from secondary school (VWO, profile Nature and Health) at Sint Maartenscollege in Maastricht in 2002, she started her study in Food Technology at Wageningen University, specialising in Product Functionality. Her minor thesis was performed at the Food Physics Group. Her major thesis was performed at the Laboratory of Food Chemistry, focusing on the *in vitro* fermentability of dextran oligosaccharides. As part of the MSc study programme, Annemieke spent one semester at University College Cork, Ireland. Her internship was performed at Campbell Soup Company in Utrecht at the R&D department.

After graduating in 2008, she was working as research assistant at the Laboratory of Food Chemistry commissioned by Royal Nedalco, analysing feed stocks for second generation bio ethanol. In January 2009 she started her PhD at the Laboratory of Food Chemistry within the framework of the Carbohydrate Competence Centre (CCC), of which the results are discussed in this thesis.

List of publications

Pustjens, A. M., De Vries, S., Gerrits, W. J. J., Kabel, M. A., Schols, H. A., & Gruppen, H., Residual carbohydrates from in vitro digested processed rapeseed (*Brassica napus*) meal, *Journal of Agricultural and Food Chemistry* **2012***, 60*(34), 8257-8263.

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Pustjens, A.M., De Vries, S., Bakuwel, M., Gruppen, H., Gerrits, W.J.J., Kabel, M.A., Residual non-starch polysaccharides from (processed) rapeseed (*Brassica napus*) meal in the pig's digestive tract, *Submitted for publication.*

Pustjens, A.M., De Vries, S., Schols, H.A., Gruppen, H., Gerrits, W.J.J., Kabel, M.A., Carbohydrate structures resistant to fermentation in broilers fed (processed) rapeseed (*Brassica napus*) meal, *Submitted for publication*.

Overview of completed training activities

Discipline specific activities

Summerschool glycosciences† (VLAG), Wageningen, The Netherland, 2010
CCC-scientific days†‡, Groningen, The Netherlands, 2010, 2011, 2012, 2013
Plant cell wall meeting, Porto, Portugal, 2010
Advanced food analysis† (VLAG), Wageningen, The Netherlands, 2010
Fibres for monogastrics, Boxmeer, The Netherlands, 2011
Chromeleon gebruikersdag, Heeze, The Netherlands, 2011
Food and biorefinery enzymology† (VLAG), Wageningen, The Netherlands, 2011
Plant and seaweed workshop‡, Nantes, France, 2012

General courses

PhD introduction week (VLAG), Maastricht, The Netherlands, 2009
PhD competence assessment, Wageningen, The Netherlands, 2009
Afstudeervak organiseren en begeleiden, Wageningen, The Netherlands, 2009
Techniques for writing and presenting a scientific paper, Wageningen, The Netherlands, 2010
Basic statistics, Wageningen, The Netherlands, 2011
Career perspectives, Wageningen, The Netherlands, 2012

Additional activities

Preparation PhD research proposal, Wageningen, The Netherlands, 2009
Food Chemistry study trip, Ghent, Belgium, 2009
Food Chemistry PhD-trip†‡, Switzerland and Italy, 2010
Food Chemistry PhD-trip†‡, Malaysia and Singapore, 2012
Food Chemistry seminars, Wageningen, The Netherlands, 2009-2013
Food Chemistry colloquia, Wageningen, The Netherlands, 2009-2013

† Poster presentation
‡ Oral presentation

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