Fibre degradation by pig microbiota

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Fibre degradation by pig microbiota

Cheng Long

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Fibre degradation by pig microbiota

Dissertation

to obtain the degree of Doctor at Maastrich University,
on the authority of the Rector Magnificus Prof.dr. Rianne M. Letschert
in accordance with the decision of the Board of Deans,
to be defended in public on Thursday 26th of November 2020 at 16.00 hours

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Abstract

The aim of the thesis was to investigate whether degradation of rapeseed meal (RSM) by a swine gut microbiota consortium was improved by modifying RSM by treatment with cellulase (CELL), two pectinases (PECT), or alkaline (ALK) compared to untreated RSM and to assess whether microbiota composition and activity changed. The processed RSM was subjected to gastric and small intestinal digestion as well. First, the *Swine Large Intestinal in vitro Model* (SLIM) was developed, which was developed based on the human, computer-controlled, dynamic TNO *in vitro* model of the colon, nick-named TIM-2. Second, untreated and pre-digested and processed RSM was fermented in SLIM by a standardized pig gut microbiota. A shot of 5 g RSM (modified or not) was directly fed to the standardized swine gut microbiota after the system adaption period, and the effect of processing methods on the RSM utilization and microbiota composition was studied. The results showed that ALK, CELL, PECT1, and PECT2 changed microbial community composition, increased the predicted abundance of microbial fibre-degrading enzymes and pathways, and increased acetic acid, propionic acid, butyric acid, and total SCFA production. The increased microbial genera positively correlated with SCFA production. Monoclonal antibody (mAb) analyses showed that the cell wall polysaccharide structures of RSM shifted after ALK, CELL, PECT1, and PECT2 treatment. The degradation of NSP during the fermentation period was dynamic, and not continuous based on the epitope recognition by mAbs. Next, a standardized swine gut microbiota was fed for 48 h with pre-digested RSM. Adaptive gPCA showed that CELL and ALK had larger effects on the microbiota composition than PECT1 and PECT2, and all substrates had larger effects than CON (untreated RSM). The relative abundance of family Prevotellaceae was significantly higher in CELL treatment compared to others. *Prevotella* 9 had significant positive correlations with propionic and valeric acid, and *Mogibacterium* positively correlated with acetic and caproic acid. Subsequently, The swine gut microbiota was adapted to modified RSM in SLIM for 48 h first, which was followed by a shot of 5 g modified RSM as evaluation period. The predicted relative abundances of carbohydrate digestion and absorption, glycolysis, pentose phosphate pathway, and pyruvate metabolism were significantly increased upon CELL and ALK feeding, and CELL and ALK also exhibited increased total short-chain fatty acid (SCFA) production compared to CON. *Megasphaera*, *Prevotella*, and *Desulfovibrio* were significantly positively correlated with SCFA production. Findings were validated in ileal cannulated pigs, which showed that CELL and ALK increased fibre degradation of RSM. Last, a preliminary novel high-throughput technique to screen polysaccharide structures, as a tool to further study fibre degradation by the (pig) gut microbiota, was developed. The technique was developed with the use of carbohydrate binding modules (CBMs) with unique specificities for plant cell wall polysaccharides, linked to a green fluorescent protein (GFP) as reporter.

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Chapter 1

General introduction

Partly submitted for publication:

Long, C., K. Venema. Chemical and nutritional characteristics, and microbial degradation of rapeseed meal recalcitrant carbohydrates. Submitted for publication.

1.1 Background

The European Union (EU) is the second-largest importer of raw material for feed (especially protein-rich feed ingredients) in the world, after China. Therefore, in order to have a more sustainable supply of responsible protein-rich ingredients, the European livestock sector needs an alternative local protein feed ingredient to fill the “protein gap”.

Rapeseed meal (RSM), a byproduct from rapeseed oil production, is not only a suitable protein source for animal feed but also a potential energy source. RSM contains a high amount of cell wall polysaccharides, even higher when compared to soybean meal commonly used in the feed industry. However, the high amount of pectins in RSM can also reduce the absorption of other nutrients in the gut. Therefore, the aim of the project was to improve utilization of recalcitrant fibre of by-product of rapeseed oil. Enzymatic and chemical treatment on the RSM were performed in the project to improve the digestibility and fermentability of the by-product. This thesis deals with the pig microbiota.

As fibres are fermented in the large intestine by the gut microbiota, this thesis focused on the interaction between gut microbiota and modified RSM, and energy yield from RSM *in vitro* and *in vivo* (cannulated pigs). It was expected that our findings could help to guide swine industry with their feed additive strategies to improve efficiency and productivity, through the indication of how feed enzymes may modulate microbial status, which in turn may provide good opportunity to develop novel carbohydrase particularly in swine feed.

1.2 Characterization of RSM carbohydrates

Carbohydrates in rapeseed meal (*B. napus*) are mainly pectins and (hemi)celluloses, and carbohydrates comprise 35 to 36% of the RSM dry matter (DM) (1, 2). The common feedstuff analysis shows commercial RSM contains 12.1% crude fiber and 34% nitrogen free extract (NFE) (Table 1). Carbohydrates of RSM can be categorized into non-structural carbohydrates and structural carbohydrates: a portion of the NFE is non-structural carbohydrates, whereas the crude fiber and the remainder of the NFE are structural polysaccharides. The composition of the different categories of RSM-carbohydrates are displayed in Table 1.

Table 1. Carbohydrates composition of rapeseed meal (oil-free dry matter)^a.

Component, %	RSM
Crude fiber	12.1
Ether extract	3.8 to 4.1
Acid detergent fiber	17.3
Neutral detergent fiber	22.7
Lignin	2.6
Non-structural carbohydrates ^b	3.2
Structural carbohydrates	29 ^c
Nitrogen free extract	34
Monosaccharides composition, mol %	
Glucose	40
Arabinose	17
Galactose	10
Xylose	9
Fructose	1
Mannose	2
Rhamnose	2
Uronic acid	20
Water-soluble carbohydrates	18.7
Monosaccharides and sucrose	16.7
Polysaccharides	2.0
Insoluble carbohydrates	15.8
Carbohydrate total	34.5-36

^a adapted from reference (1, 3-5)^b Fructose, glucose, galactose, sucrose, galactinol, raffinose and stachyose.^c Pectins, cellulose residue, amylose, arabinan, arabinogalactan.

1.2.1 Non-structural carbohydrates

The non-structural carbohydrates in RSM are comprised of low molecular weight sugars, oligosaccharides and storage polysaccharides.

1.2.1.1 Low molecular weight sugars

Low molecular weight sugars mainly are sucrose (2.3 to 2.9%), fructose (0.05 to 0.16%), and glucose (0.05 to 0.16%) (3).

1.2.1.2 Oligosaccharides

The primary oligosaccharides found in RSM are raffinose, stachyose, galactinol, and myo-inositol. Of those, stachyose has the highest concentration (0.4 to 0.5 %), followed by raffinose (0.05 to 0.16 %), galactinol (0.1 %) and myo-inositol (0.1 %) (6).

1.2.1.3 Storage polysaccharides

Only low concentrations of storage polysaccharides are present in RSM. The primary storage polysaccharide is starch. The starch content of the seed approaches 50% during early development, but starch almost completely disappears when energy stores are converted into oil as the seed matures. Rommi et al. (2) reported that starch concentrations found in the intact was the same as dehulled rapeseed press cakes (0.2% of DM), while the starch content of canola meal was up to 2.5% of DM (7). Starch granules are comprised of two main macromolecules (amylose and amylopectin). Amylose is a linear polymer consisting of α -1,4-linked D-glucose units (GU), and amylose includes 500-600 GU in its native form, which can be subdivided into 1-20 chains. Amylopectin is a large branched macromolecule (molecular weight 10^7 - 10^9 kDa), with approximately 5% of its total linkages consisting of β -1,6-linked GU plus a large number of short α -1,4-linked linear GU chains (12-70 GU) (8).

1.2.2 Structural polysaccharides

The rest of the RSM carbohydrates is made up of structural polysaccharides. These include

cellulose, hemicellulose (xyloglucan and xylan), and pectic polysaccharides (homogalacturonan, rhamnogalacturonan I, arabinan, and arabinogalactan).

1.2.2.1 Polysaccharides composition

RSM contains 34.5 % NSP of its DM, and RSM is high in glucosyl (40 mol%), arabinosyl (19 mol%) and uronyl residues (18 mol%) (1, 9). Water-soluble and water-insoluble carbohydrates comprise 18.7 and 15.8 % of the total carbohydrates content of RSM, respectively (Table 1). Water-soluble carbohydrates mainly contain glucosyl (64 mol%) and some galactosyl residues (17 mol%) (1), while water-insoluble carbohydrates are mainly glucosyl (32 mol%), arabinosyl (25 mol%), uronyl (18 mol%) and xylosyl residues (12%) (Table 2).

The detailed monosaccharide constituent compositions of the Water unextractable solids (WUS), which were sequentially extracted with chelating agent (ChSS) to release calcium-bound pectins, dilute alkali (DASS) to release pectins tightly bound to hemicellulose, 4 and 6 molar alkali (4 MASS and 6 MASS) to release hemicelluloses, and cellulose that will remain in the residue (RES), are presented in Table 2. The composition of the glycosidic residues indicates the presence of 1,5-linked arabinan branched at O-2, galactomannan, homogalacturonan, rhamnogalacturonan I, type II arabinogalactan, glucuronoxylan, XXGG-type and XXXG-type xylo(X)glucan(G), and cellulose in RSM (1, 2, 10). Arabinan consists of a linear backbone of α -L-1,5-linked arabinose (Ara) units with α -1,2-lined or α -1,3-lined Ara units (Figure 1). Galactomannan has a backbone of β -1,4-linked mannose units, substituted with α -1,6-lined galactose units (Figure 1). Homogalacturonan (HG) is the simplest form of pectin, consisting of a linear chain of α -1,4-linked galacturonic acid, and part of its carboxyl groups is esterified with acetyl or methyl groups (Figure 1). Rhamnogalacturonan I (RG-I) consists of alternating rhamnose (Rha) and galacturonic acid (GalA) residues [-₂)- α -L-Rha-(1,4)- α -D-GalA-(1,-)], which is highly ramified with single terminal β -D-Gal and/or α -D-Ara at position O-4 or O-3 of the rhamnosyl residues where the α -D-GalA residues are often O-acetyl esterified at O-2 and/or O-3 (Figure 1) (11). Type II arabinogalactan (AG) is composed of a β -1,3-galactan backbone and β -1,6-galactan side chains (Figure 1). The side chains are variably decorated with L-arabinose. Glucuronoxylan (GX) has a linear backbone of β -(1-4)-linked D-xylosyl (Xyl) residues, which can be ramified with acetyl and arabinosyl residues, and some of the Xyl residues are decorated with a single α -D-glucuronic acid (GlcA) or 4-O-methyl-D-glucuronic acid (MeGlcA) residue at O2 (Figure 1) (12, 13). Both XXGG- and XXXG-type xyloglucan (XG) exist in RSM. XXGG (Figure 1) consists of a β -1,4-linked D-glucosyl (Glc) backbone carrying various side chains of D- β -1,2-Gal-D- α -1,6-Xyl and L- α -1,2-Ara-D- β -D- α -1,6-Xyl (14), whereas, XXXG (Figure 1) comprises a β -1,4-linked D-glucosyl backbone carrying continuous side chains of D- α -1,6-Xyl, D- β -1,2-Gal-D- α -1,6-Xyl, and L- α -1,2-Ara-D- β -1,2-Gal-D- α -1,6-Xyl in every 4 residues of D- β -1,4-Glc (14). Cellulose consists of long linear chains of β -1,4-linked D-Glc residues, with a degree of polymerization between 2000-14000 residues (Figure 1).

Table 2. Molar sugar composition of RSM fractions^a

	Molar composition (mol%)						
	Rha	Ara	Xyl	Man	Gal	Glc	UA
RSM	2	19	8	6	10	40	15
WSS	Tr ^b	7	1	7	17	64	5
WUS	1	25	12	4	8	32	18
CHSS	2	15	4	2	4	3	71
DASS	Tr	44	6	3	10	9	29
4MASS	Tr	17	20	15	13	30	5
6MASS	1	29	22	2	13	23	11
RES	4	10	6	4	6	40	20

^a adapted from reference (1)^b Trace amounts.

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; Ara: arabinose; Xyl: xylose; Man: mannose; Fuc: fucosyl; Gal: galactose; Glc: glucose; UA: uronic acids.

Figure 1. Schematic representation of structures of polysaccharides in RSM.

The backbone of these polysaccharides can also be esterified by methyl-esters, ethyl-esters, and glycosyls (arabinosyl, galactosyl, mannosyl, glucosyl, xylosyl, or frucosyl).

Except for containing glucosyl residues, RES (Table 2) also has quite some arabinosyl and uronyl residues (10% and 20%, respectively), which indicates that some pectic polysaccharides are tightly associated with cellulose microfibrils. This suggests that the cell wall polysaccharide matrix of rapeseed meal is strongly interlinked. The specific structures of dietary fibers of RSM are still not entirely understood. The main restriction may be the analysis method, but nowadays, the comprehensive microarray polymer profiling (CoMPP)

technique is a powerful tool for probing cell wall structure studies (15). The profiles generated by CoMPP provide a global snapshot of cell-wall composition. It cannot only detect the amount of the particular polysaccharides but also their linkage type.

1.2.2.2 Glycosidic linkage type

A covalent link between one carbohydrate molecule and a second carbohydrate molecule is called a glycosidic bond. Glycosidic linkage is the type of bond between two adjacent glycosides in the chain of polysaccharides (16).

The proposed sugar linkage compositions of RSM fractions are shown in Table 3. The data can only be used in a qualitative manner instead of quantitative, due to the poor DMSO solubility of the fractions and the high amount of uronic acids (which are not detected with this method) present in some samples (17).

Table 3. Sugar linkage composition of RSM fractions (mol%)^a

	WUS	DASS	4 MASS	6 MASS
t-Ara ^b	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 ^c	1.10	1.15	1.04	1.36

^a adapted from reference (2)

^b t: terminal.

^c T/B: ratio terminally linked residues: branching points.

Ara: arabinose; Xyl: xylose; Man: mannose; Fuc: fucosyl; Gal: galactose; Glc: glucose; UA: uronic acid.

1.3 Microbial degradation of RSM cell wall carbohydrates

Low molecular weight sugars and starch can be 100% digested, while (oligo- and poly-)saccharides are considered indigestible in the small intestine due to lack of the necessary enzymes in monogastric animals (18). Indeed, mammalian genomes do not encode most of the enzymes needed to degrade the structural polysaccharides present in plant material. Instead, a complex mutual dependence has developed between the mammalian host and symbiotic gut microorganisms that do possess the ability to access the abundant source of energy in carbohydrate that are indigestible by the host. The gut microbiota has glycoside hydrolases (GH) that are able to degrade the oligo- and polysaccharides into small oligomers and monosaccharides which are subsequently taken up and fermented.

1.3.1 Glycoside hydrolases

Glycoside hydrolases (GHs) are a vast repertoire of cell wall-degrading enzymes that hydrolyse glycosidic bonds between two or more carbohydrate modules or a sugar and a non-sugar moiety within carbohydrates or oligosaccharides (19, 20). GH families widely exist in prokaryotic, eukaryotic and archaea species. A total of 167 GH families have been identified until now (accessed on July-2020, <http://www.cazy.org/Glycoside-Hydrolases.html>).

Carbohydrate binding modules (CBMs) are the non-catalytic part of cell-wall-degrading enzymes, and they are attached to the GH catalytic modules. Usually, CBMs have to recognize and bind to the specific polysaccharides first, before the GHs cleave the polysaccharides (21).

1.3.2 Arabinan

Arabinan can be hydrolyzed by α -L-Arabinofuranosidases (EC 3.2.1.55) and endo- α -1,5-L-arabinanases (EC 3.2.1.99), which are found in GH families 3, 43, 51, 54 and 62, and which release arabinosyl oligomers and L-arabinose (22). A previous study reported that *Bacillus subtilis* contained a series of arabinan-degrading genes, *abnA*, *abn2*, *abfA*, and *abf2*, which were induced by arabinose and arabinan, repressed by glucose, and subjected to temporal regulation (23). *AbnA* and *abn2* can express extracellular endo- α -1,5-L-arabinanases belonging to GH43, which hydrolyzes arabinan (branched) and linear α -1,5-L-arabinan and produces arabinose and arabino-oligosaccharides (24). The resulting products, arabinose and arabino-oligosaccharides, are transported into the cell by different transport systems. Arabinose enters the cell mainly through the *araE* permease (25), and the uptake of arabinose oligomers occurs most likely via *araNPQ*, and ABC-type transporter (26). These products are further digested into the monosaccharide arabinose by two intracellular arabinofuranosidases, *abfA* and *abf2*, which are α -L-arabinofuranosidases (EC 3.2.1.55) belonging to GH51. *AbfA* acts preferentially on (1 $\rightarrow$ 5) arabinofuranosyl linkages, and in contrast *abf2* is most active on (1 $\rightarrow$ 2) and (1 $\rightarrow$ 3) linkages (27). After this, L-arabinose is converted into D-xylulose-5-phosphate, which is further catabolized through the pentose phosphate pathway. The induction mechanism of these genes is mediated through negative control by the key regulator of arabinose metabolism, *araR*. The transcriptional repression of the *abfA* and *abf2* genes is achieved by a tightly controlled mechanism but the regulation of *abnA* is more flexible.

The presence of α -L-arabinofuranosidases (Table 4) has been determined in *Bifidobacterium longum* subsp. *Longum* (28), *Bacteroides thetaiotaomicron* VPI-5482 (29), *Sulfolobus solfataricus* P2 (30), *Anoxybacillus kestanbolensis* AC26Sari (31), *Monoglobus pectinilyticus* (32) and *Roseburia faecis* M72/1 (33). Endo- α -1,5-L-arabinanases (Table 4) are present in *Paenibacillus polymyxa* (34), *Bacillus licheniformis* (35), *Caldicellulosiruptor saccharolyticus* (36), *Bacillus subtilis* (37), *Bacillus. Thermodenitrificans* (38), *Pseudomonas fluorescens* subsp. *cellulosa* (39), *Monoglobus pectinilyticus* (32) and *Roseburia faecis* M72/1(33).

1.3.3 Galactomannan

Endo-1,4- β -mannanase (EC 3.2.1.78), β -mannosidase (EC 3.2.1.25) and α -galactosidase (EC 3.2.1.22) are involved in degradation of galactomannan into monosaccharides (40). β -mannanase degrades randomly within the main chain of galactomannans, and produces shorter galactomanno-oligosaccharides that can be further hydrolyzed by β -mannosidase and α -galactosidase. β -mannosidase hydrolyses β -1,4-linked mannose residues from the nonreducing end of the galactomanno-oligosaccharides and α -galactosidase hydrolyses terminal α -1,6-linked galactose residues from galactomannans or the galactomanno-oligosaccharides. The gut microbes *Bacillus subtilis* YH12 (41), *Bacillus subtilis* TD7 (42),

Bacillus subtilis Bs5 (43), *Bacillus licheniformis* DSM13 (44), *Sphingomonas* sp. JB13 (45), *Sphingobacterium* sp. GN25 (46), *Klebsiella oxytoca* KUB-CW2-3 (47), *Enterobacter* sp. strain N18 (48), *Flavobacterium* sp (49), *Pseudomonas cellulosa* (39), *Monoglobus pectinilyticus* (32) and *Bacteroides ovatus* (50) are reported to have endo-1,4- β -mannanase (Table 4). While *Bifidobacterium* sp. (51), *Bifidobacterium longum* subsp. *longum* NCC2705 (52), *Bacteroides ovatus* (53, 54), *Cellvibrio mixtus* (55), *Bacteroides thetaiotaomicron* (56), *Pseudomonas cellulose* (39), *Kitasatospora* sp. (57) are reported to have β -mannosidase (Table 4). Moreover, *Lactobacillus acidophilus* NCFM (58), *Lactobacillus crispatus* ST1 (59), *Lactobacillus brevis* (60), *Bifidobacterium animalis* subsp. *lactis* BI-04 (58), *Arthrobacter* sp. C2-2 (61), *Bacillus megaterium* (62), *Dictyoglomus thermophilum* (63), *Bacillus stearothermophilus* NCIM-5146 (64), *Bacillus stearothermophilus* NUB 3621 (65), *Bacteroides ovatus* 0038-1 (66), *Bifidobacterium bifidum* NCIMB41171 (67), *Bifidobacterium adolescentis* DSM20083 (68), *Bifidobacterium breve* 203 (69), *Clostridium stercorarium* (70) and *Monoglobus pectinilyticus* (32) are reported to have α -galactosidases (Table 4).

Asperigillus niger contains galactomannan-degradation genes, *aglA*, *aglB* and *aglC* (encoding α -galactosidases), and *mndA* (encoding a β -mannosidase). *AgIA* and *aglB* have been classified into GH27, while *aglC* has been classified into GH36, and *mndA* belongs to GH2. The metabolism mechanism of galactomannan is that α -galactosidase (encoded by *aglC*) and β -mannosidase (encoded by *mndA*) hydrolyze the galactomannan to the oligosaccharide Gal2Man5 (monomeric sugars), where *mndA* cleaves single mannose units from the nonreducing end of the substrate until it reaches a galactose side-group (71), and afterward the nonreducing galactose group is hydrolyzed by α -galactosidase (*aglA*, *aglB*, or *aglC*). *AgIB* and *mndA* play a major role in the degradation of galactomannan in *A. niger*. The expression of *aglA* is high on galactose and galactose-containing oligosaccharides but is fully repressed in the presence of glucose (72). Little is known about genes and their regulation in members of the gut microbiota.

1.3.4 Homogalacturonan

Homogalacturonan (HG) can be cleaved by α -1,4-L-galacturonan reducing-end-disaccharide-lyase (pectate disaccharide-lyase) (EC 4.2.2.9), (1 $\rightarrow$ 4)-6-O-methyl- α -D-galacturonan lyase (pectin lyase) (EC 4.2.2.10), (1 $\rightarrow$ 4)- α -D-galacturonan reducing-end-trisaccharide-lyase (pectate trisaccharide-lyase) (EC 4.2.2.22), α -1,4-D-galacturonan lyase (pectate lyase) (EC 4.2.2.2), and (1 $\rightarrow$ 4)- α -D-galacturonan glycanohydrolase (endopolygalacturonase) (EC 3.2.1.15) (73-78). Pectin lyase (EC 4.2.2.10) provides cleavage of α -1,4-linked D-galacturonan methyl ester to give oligosaccharides with 4-deoxy-6-O-methyl- α -D-galact-4-enuronosyl groups at their non-reducing ends, while α -1,4-D-galacturonan lyase (EC 4.2.2.2) cleaves α -1,4-linked D-galacturonan to give oligosaccharides with 4-deoxy- α -D-galact-4-enuronosyl groups at their non-reducing ends. Afterwards, pectate disaccharide-lyase (EC 4.2.2.9) hydrolyzes these oligosaccharides to (1,4- α -D-galacturonosyl)_{n-2} and 4-(4-deoxy- α -D-galact-4-enuronosyl)-D-galacturonate, and (1,4- α -D-galacturonosyl)_{n-2} will be cleaved by pectin lyase (EC 4.2.2.10 or EC 2.2.2) again until the disaccharide. Polygalacturonase (EC 3.2.1.15) randomly hydrolyzes (1 $\rightarrow$ 4)- α -D-galactosiduronic linkages in pectate and other galacturonans. Pectate disaccharide-lyase (Table 4) has been reported in *Caldicellulosiruptor bescii* (79), *Bacteroides thetaiotaomicron* (80), *Bacteroides ovatus* (81), *Bacillus pumilus* BK2 (82), *Eubacterium eligens*, *Faecalibacterium prausnitzii* (83) and *Monoglobus pectinilyticus* (32). Pectate lyase (Table 4) has been reported in *Caldicellulosiruptor bescii* (79), genus *Bacillus*: *Bacillus subtilis*, *B.*

licheniformis, *B. cereus*, *B. circulans*, *B. pasteurii*, *B. amyloliquefaciens*, and *B. pumilus* (84, 85), *Paenibacillus* sp. (86), *Clostridium cellulovorans* (87), *Streptomyces thermocarboxydus* (88), *Bacteroides thetaiotaomicron* (80), *Bacteroides ovatus* (81), *Eubacterium eligens*, *Faecalibacterium prausnitzii* (83) and *Monoglobus pectinilyticus* (32). Endopolygalacturonase (Table 4) has been reported in *Caldicellulosiruptor bescii* (79), *Bacteroides thetaiotaomicron* (80), *Bacteroides ovatus* (81), *Eubacterium eligens*, *Faecalibacterium prausnitzii* (83), *Monoglobus pectinilyticus* (32) and *Bifidobacterium longum* subsp. *Longum* (89).

1.3.5 Rhamnogalacturonan I

Utilization of rhamnogalacturonan I (RG-I) by microbes is mediated by a series of enzymes, which is well studied in *Bacillus subtilis* (90). Two main enzymes, a hydrolase and a lyase, are involved in the degradation of the RG-I backbone, whereas a few other enzymes are responsible for the breakdown of the RG-I side chains. Rhamnogalacturonan hydrolase (rhamnogalacturonan α -L-rhamnopyranohydrolase) cleaves α -1,2 linkages between GalA and Rha (91). Rhamnogalacturonan lyase [α -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- α -D-galactopyranosyluronate endolyase (EC 4.2.2.23)] cleaves the α -1,4 linkages of RG-I resulting in a double bond in nonreducing GalA residue (92). Rhamnogalacturonan lyase and hydrolase (Table 4) have been reported in *Caldicellulosiruptor bescii* (79), *Bacteroides thetaiotaomicron* (80), *Bacteroides ovatus* (81), *Bacillus subtilis* (93), *Bacillus licheniformis* (94), *Cellvibrio japonicus* (95), *Clostridium cellulolyticum* (96), *Bacillus licheniformis* DSM13 (94), *Pseudomonas cellulose* (95), *Penicillium chrysogenum* (97) and *Monoglobus pectinilyticus* (32).

1.3.6 Type II arabinogalactan

Exo- β -1,3-galactanase (EC 3.2.1.145) cleaves the β -1,3-galactan backbone of type II arabinogalactan via bypassing the β -1,6-galactan side chains and releasing β -1,6-galactooligosaccharides and their derivatives (98). The β -1,6-galactan side chains are hydrolyzed to β -1,6-galactooligosaccharides of various degrees of polymerization (DP) by endo- β -1,6-galactanase (EC 3.2.1.164) (99, 100). On the other hand, exo- β -1,6-galactobiohydrolase releases β -1,6-galactobiose (β -1,6-Gal2) from the nonreducing terminal end of β -1,6-galactooligosaccharides, and the α -L-arabinofuranosidase (EC 3.2.1.55) releases arabinofuranose (Araf) from α -1,3-Araf-substituted β -1,6-galactooligosaccharides (101). Exo- β -1,3-galactanase (Table 4) has been reported in *Monoglobus pectinilyticus* (32), *Bifidobacterium longum* subsp. *longum* (89), *Clostridium thermocellum* (102), *Phanerochaete chrysosporium* (103), *Sphingomonas* sp. (104), *Bacteroides thetaiotaomicron* (105), *Bacteroides ovatus* (105), *Bacteroides caccae* (106) and *Bacteroides cellulosilyticus* (106). Endo- β -1,6-galactanase (Table 4) had been reported in *Streptomyces avermitilis* NBRC14893 (107), *Bacteroides ovatus* (105), *Bacteroides caccae* (106), *Bacteroides cellulosilyticus* (106) and *Bifidobacterium longum* subsp. *longum* (89). Exo- β -1,6-galactobiohydrolase (Table 4) has been reported in *Monoglobus pectinilyticus* (32), *Bacteroides thetaiotaomicron* (106), *Streptomyces avermitilis* (107), *Bacteroides ovatus* (105), *Bacteroides caccae* (106), *Bacteroides cellulosilyticus* (106) and *Bifidobacterium longum* subsp. *Longum* (89).

1.3.7 Glucuronoxylan

Two enzymes, β -(1-4)-D-xylan xylanohydrolase (endo- β -1,4 xylanase) (EC 3.2.1.8) and 1,4- β xylohydrolase (exo- β -1,4 xylanase) (EC 3.2.1.37), are involved in degrading the β -1,4 xylosyl linkages in unsubstituted domains along the xylan backbone of glucuronoxylan (GX) (108, 109). Glucuronoxylanase cleaves glucuronosyl moieties which are substituted as

monomeric side chains on the xylan backbone (110). Endo- β -1,4-xylanase (Table 4) has been reported in *Pseudomonas boreopolis* G22 (111), *Bacteroides ovatus* (80), *Monoglobus pectinilyticus* (32), *Bacteroides thetaiotaomicron* (105), *Bacteroides caccae* (106), *Bacteroides cellulosilyticus* (106), *Clostridium thermocellum* (112), *Bacillus subtilis* (113) and *Streptomyces turgidiscabies* (114). Exo- β -1,4 xylanase (Table 4) has been reported in *Monoglobus pectinilyticus* (32), *Luteimicrobium xylanilyticum* (115), *Amycolatopsis mediterranei* (115), *Clostridium thermocellum* (112), *Bacillus subtilis* (113) and *Streptomyces turgidiscabies* (114).

1.3.8 Xyloglucan

A set of glucanases and glycosidases are involved in cleaving xyloglucan (XG) into monosaccharides by two-step degradation (116, 117). Endo- β -1,4-glucanase (EC 3.2.1.4) hydrolyzes XG into large fragments, which are further degraded into monosaccharides by α -xylosidase (EC 3.2.1.177) and β -glucosidase (EC 3.2.1.21) (118). Endo- β -1,4-glucanase (Table 4) has been reported in *Monoglobus pectinilyticus* (32), *Caldicellulosiruptor kronotskyensis* (119), *Roseburia* sp (33), *Eubacterium rectale* group (33), *Ruminococcus champanellensis* (120), *Ruminococcus bromii* (121), *Ruminiclostridium cellulolyticum* (122) and *Phaeoacremonium minimum* (123). Exo- β -1,4 xylanase (Table 4) has been reported in *Monoglobus pectinilyticus* (32), *Luteimicrobium xylanilyticum* (115), *Amycolatopsis mediterranei* (115), *Clostridium thermocellum* (112), *Bacillus subtilis* (113), *Streptomyces turgidiscabies* (114), *Ruminiclostridium cellulolyticum* (122) and *Phaeoacremonium minimum* (123). While α -D-xylosidase (Table 4) has been reported in *Sulfolobus solfataricus* P2 (30), *Talaromyces thermophilus* (124), *Cellvibrio japonicus* (125), *Bacteroides thetaiotaomicron* (80), *Bacteroides ovatus* (80) and *Monoglobus pectinilyticus* (32). Moreover, β -glucosidase (Table 4) has been reported in *Bifidobacterium adolescentis* (126), *Bacteroides ovatus* (127), *Listeria innocua* (128), *Streptomyces venezuelae* (129), *Pyrococcus furiosus* (130), *Cellvibrio japonicus* (125), *Caldicellulosiruptor saccharolyticus* (131), *Microbispora bispora* (132), *Thermoanaerobacter brockii* (133), *Thermobifida fusca* (111), *Pseudomonas* sp. (134), *Monoglobus pectinilyticus* (32), *Ruminococcus champanellensis* (120) and *Ruminococcus bromii* (121).

1.3.9 Cellulose

The enzymes of the cellulase system consist of endo- β -1,4-glucanase (EC 3.2.1.4), exo- β -1,4-glucanase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21). Endo- β -1,4-glucanase and exo- β -1,4-glucanase cleave cellulose to celloextrins and cellobiose, which are then degraded to glucose by β -glucosidase (135, 136). Endo and exo- β -1,4-glucanase (Table 4) have been reported in *Monoglobus pectinilyticus* (32), *Caldicellulosiruptor kronotskyensis* (119), *Roseburia* sp (33), *Eubacterium rectale* group (33), *Ruminococcus champanellensis* (120), *Ruminococcus bromii* (121), *Ruminiclostridium cellulolyticum* (122), *Paenibacillus* sp (137) and *Phaeoacremonium minimum* (123). The enzyme β -glucosidase (Table 4) has been reported in *Bifidobacterium adolescentis* (126), *Bacteroides ovatus* (127), *Listeria innocua* (128), *Streptomyces venezuelae* (129), *Pyrococcus furiosus* (130), *Cellvibrio japonicus* (125), *Caldicellulosiruptor saccharolyticus* (131), *Microbispora bispora* (132), *Thermoanaerobacter brockii* (133), *Thermobifida fusca* (111), *Pseudomonas* sp. (134), *Monoglobus pectinilyticus* (32), *Ruminococcus champanellensis* (120) and *Ruminococcus bromii* (121).

Table 4. Gut microbes containing the plant cell wall degrading enzymes

Microbial enzymes	Bacteria
α -L-arabinofuranosidases	<i>Bifidobacterium longum</i> subsp. <i>Longum</i> (28), <i>Bacteroides thetaiotaomicron</i> VPI-5482 (29), <i>Sulfolobus solfataricus</i> P2 (30), <i>Anoxybacillus kestanbolensis</i> AC26Sari (31), <i>Monoglobus pectinilyticus</i> (32) and <i>Roseburia faecis</i> M72/1 (33)
endo- α -1,5-L-arabinanases	<i>Paenibacillus polymyxa</i> (34), <i>Bacillus licheniformis</i> (35), <i>Caldicellulosiruptor saccharolyticus</i> (36), <i>Bacillus. Subtilis</i> (37), <i>Bacillus. Thermodenitrificans</i> (38), <i>Pseudomonas fluorescens</i> subsp. <i>cellulosa</i> (39), <i>Monoglobus pectinilyticus</i> (32) and <i>Roseburia faecis</i> M72/1(33)
endo-1,4- β -mannanase	<i>Bacillus subtilis</i> YH12 (41), <i>Bacillus subtilis</i> TD7 (42), <i>Bacillus subtilis</i> Bs5 (43), <i>Bacillus licheniformis</i> DSM13 (44), <i>Sphingomonas</i> sp. JB13 (45), <i>Sphingobacterium</i> sp. GN25 (46), <i>Klebsiella oxytoca</i> KUB-CW2-3 (47), <i>Enterobacter</i> sp. strain N18 (48), <i>Flavobacterium</i> sp (49), <i>Pseudomonas cellulosa</i> (39), <i>Monoglobus pectinilyticus</i> (32) and <i>Bacteroides ovatus</i> (50)
β -mannosidase	<i>Bifidobacterium</i> sp. (51), <i>Bifidobacterium longum</i> subsp. <i>longum</i> NCC2705 (52), <i>Bacteroides ovatus</i> (53, 54), <i>Cellvibrio mixtus</i> (55), <i>Bacteroides thetaiotaomicron</i> (56), <i>Pseudomonas cellulose</i> (39), <i>Kitasatospora</i> sp. (57)
α -Galactosidases	<i>Lactobacillus acidophilus</i> NCFM (58), <i>Lactobacillus crispatus</i> ST1 (59), <i>Lactobacillus brevis</i> (60), <i>Bifidobacterium animalis</i> subsp. <i>lactis</i> BI-04 (58), <i>Arthrobacter</i> sp. C2-2 (61), <i>Bacillus megaterium</i> (62), <i>Dictyoglomus thermophilum</i> (63), <i>Bacillus stearothermophilus</i> NCIM-5146 (64), <i>Bacillus stearothermophilus</i> NUB 3621(65), <i>Bacteroides ovatus</i> 0038-1 (66), <i>Bifidobacterium bifidum</i> NCIMB41171 (67), <i>Bifidobacterium adolescentis</i> DSM20083 (68), <i>Bifidobacterium breve</i> 203 (69), <i>Clostridium stercorarium</i> (70) and <i>Monoglobus pectinilyticus</i> (32)
Pectate disaccharide-lyase	<i>Caldicellulosiruptor bescii</i> (79), <i>Bacteroides thetaiotaomicron</i> (80), <i>Bacteroides ovatus</i> (81), <i>Bacillus pumilus</i> BK2 (82), <i>Eubacterium eligens</i> , <i>Faecalibacterium prausnitzii</i> (83) and <i>Monoglobus pectinilyticus</i> (32)
pectate lyase	in <i>Caldicellulosiruptor bescii</i> (79), genus <i>Bacillus</i> : <i>Bacillus subtilis</i> , <i>B. licheniformis</i> , <i>B. cereus</i> , <i>B. circulans</i> , <i>B. pasteurii</i> , <i>B. amyloliquefaciens</i> , and <i>B. pumilus</i> (84, 85), <i>Paenibacillus</i> sp. (86), <i>Clostridium cellulovorans</i> (87), <i>Streptomyces thermocarboxydus</i> (88), <i>Bacteroides thetaiotaomicron</i> (80), <i>Bacteroides ovatus</i> (81), <i>Eubacterium eligens</i> , <i>Faecalibacterium prausnitzii</i> (83) and <i>Monoglobus pectinilyticus</i> (32)
endo-polygalacturonase	<i>Caldicellulosiruptor bescii</i> (79), <i>Bacteroides thetaiotaomicron</i> (80), <i>Bacteroides ovatus</i> (81), <i>Eubacterium eligens</i> , <i>Faecalibacterium prausnitzii</i> (83), <i>Monoglobus pectinilyticus</i> (32) and <i>Bifidobacterium longum</i> subsp. <i>Longum</i> (89)
rhamnogalacturonan lyase and hydrolase	<i>Caldicellulosiruptor bescii</i> (79), <i>Bacteroides thetaiotaomicron</i> (80), <i>Bacteroides ovatus</i> (81), <i>Bacillus subtilis</i> (93), <i>Bacillus licheniformis</i> (94), <i>Cellvibrio japonicus</i> (95), <i>Clostridium cellulolyticum</i> (96), <i>Bacillus licheniformis</i> DSM13 (94), <i>Pseudomonas cellulose</i> (95), <i>Penicillium chrysogenum</i> (97) and <i>Monoglobus pectinilyticus</i> (32)
exo- β -1,3-galactanase	<i>Monoglobus pectinilyticus</i> (32), <i>Bifidobacterium longum</i> subsp. <i>longum</i> (89), <i>Clostridium thermocellum</i> (102), <i>Phanerochaete chrysosporium</i> (103), <i>Sphingomonas</i> sp. (104), <i>Bacteroides thetaiotaomicron</i> (105), <i>Bacteroides ovatus</i> (105), <i>Bacteroides caccae</i> (106) and <i>Bacteroides cellulosilyticus</i> (106)
endo- β -1,6-galactanase	<i>Streptomyces avermitilis</i> NBRC14893 (107), <i>Bacteroides ovatus</i> (105), <i>Bacteroides caccae</i> (106), <i>Bacteroides cellulosilyticus</i> (106) and <i>Bifidobacterium longum</i> subsp. <i>longum</i> (89)
exo- β -1,6-galactobiohydrolase	<i>Monoglobus pectinilyticus</i> (32), <i>Bacteroides thetaiotaomicron</i> (106), <i>Streptomyces avermitilis</i> (107), <i>Bacteroides ovatus</i> (105), <i>Bacteroides caccae</i> (106), <i>Bacteroides cellulosilyticus</i> (106) and <i>Bifidobacterium longum</i> subsp. <i>longum</i> (89)
endo- β -1,4-xylanase	<i>Pseudomonas boreopolis</i> G22 (111), <i>Bacteroides ovatus</i> (80), <i>Monoglobus pectinilyticus</i> (32), <i>Bacteroides thetaiotaomicron</i> (105), <i>Bacteroides caccae</i> (106), <i>Bacteroides cellulosilyticus</i> (106), <i>Clostridium thermocellum</i> (112), <i>Bacillus subtilis</i> (113) and <i>Streptomyces turgidiscabies</i> (114)
exo- β -1,4 xylanase	<i>Monoglobus pectinilyticus</i> (32), <i>Luteimicrobium xylanilyticum</i> (115), <i>Amycolatopsis mediterranei</i> (115), <i>Clostridium thermocellum</i> (112), <i>Bacillus</i>

	<i>subtilis</i> (113) and <i>Streptomyces turgidiscabies</i> (114)
endo and exo- β -1,4-glucanase	<i>Monoglobus pectinilyticus</i> (32), <i>Caldicellulosiruptor kronotskyensis</i> (119), <i>Roseburia sp</i> (33), <i>Eubacterium rectale</i> group (33), <i>Ruminococcus champanellensis</i> (120), <i>Ruminococcus bromii</i> (121), <i>Ruminiclostridium cellulolyticum</i> (122) and <i>Phaeoacremonium minimum</i> (123)
α -D-xylosidase	<i>Sulfolobus solfataricus</i> P2 (30), <i>Talaromyces thermophilus</i> (124), <i>Cellvibrio japonicus</i> (125), <i>Bacteroides thetaiotaomicron</i> (80), <i>Bacteroides ovatus</i> (80) and <i>Monoglobus pectinilyticus</i> (32)
β -glucosidase	<i>Bifidobacterium adolescentis</i> (126), <i>Bacteroides ovatus</i> (127), <i>Listeria innocua</i> (128), <i>Streptomyces venezuelae</i> (129), <i>Pyrococcus furiosus</i> (130), <i>Cellvibrio japonicus</i> (125), <i>Caldicellulosiruptor saccharolyticus</i> (131), <i>Microbispora bispora</i> (132), <i>Thermoanaerobacter brockii</i> (133), <i>Thermobifida fusca</i> (111), <i>Pseudomonas sp.</i> (134), <i>Monoglobus pectinilyticus</i> (32), <i>Ruminococcus champanellensis</i> (120) and <i>Ruminococcus bromii</i> (121)

1.4 Processing to increase use of recalcitrant fibres

Our previous studies have already shown that physical processing technologies cannot significantly increase fibre degradability in monogastric animals (9, 138, 139). However, the utilization of feed enzymes is a promising method to improve fibre fermentability.

1.4.1 Enzymatic modification

Supplementation of cell wall degrading enzymes to improve feed efficiency for pigs gets more and more attention from the feed industry nowadays. Supplementation of cell wall degrading enzymes can remove side-chains of polysaccharides in plant cell wall, which make them more accessible to bacterial enzymes (138). In addition, cell wall degrading enzymes are able to reduce the digesta viscosity, which might affect absorption of other nutrients, by cleaving the viscous polysaccharides (e.g. pectin). In the end, carbohydrases can depolymerize polysaccharides to oligosaccharides, which have potential prebiotic effects to the host animal.

1.4.2 Chemical modification

A previous study showed that pre-processing with sulfuric acid could increase utilization of carbohydrates from lignocellulosic biomass (140). Mild acid-treated rye (together with heat treatment) was reported to improve the release of arabinosyl residues in chickens (141). Alkaline pretreatment of rapeseed meal in the feed improved its fermentation in pigs (142). Therefore, chemical treatment in feed might be a promising method to improve fibre fermentation in pigs.

1.5 Swine gut microbiota

Nowadays, it is well known that the gut microbiota plays a crucial role in the human and animal gut, which is related to the health status of the host as well as to nutrient digestibility (143, 144). Pigs are both an important meat source for human consumption and an animal model for biomedical research. Thus, there is numerous research about the pig gut microbiota (145-148). Ramayo-Caldas et al. (144) have studied fecal microbiota from 518 healthy piglets, and reported that the dominant microbial phyla are Firmicutes and Bacteroidetes, which is in agreement with other reports (149-152). The remaining phyla contain Actinobacteria, Proteobacteria, and others (144, 149-151). The microbial abundances in lower phylogenetic levels are highly variable, but the bacterial types are quite similar. For instance, at family level, the relative abundances of 11 families in the pig gut microbiota are Prevotellaceae (26.5%), Veillonellaceae (18.2%), Lachnospiraceae (16.6%), Ruminococcaceae (16.4%), Paraprevotellaceae (3.67%), S24-7 (identified as Muribaculaceae, 1.91%), Campylobacteraceae (1.24%), Clostridiaceae (1.24%), and others

(146). A similar number of family types are reported in another study (152), and in addition some additional ones that were found in that study are Streptococcaceae, Lactobacillaceae, Erysipelotrichaceae, and Coriobacteriaceae. The same findings are observed at genus level, namely that the core genera reported by different researchers are similar, but the abundances are highly variable (146, 152, 153), e.g., due to different diet intake or different pig-race. Dietary fiber and resistant starch are known to modulate the intestinal microbiota and thereby contribute to gut health status by promoting the growth of beneficial microorganism in pigs and contribute to improvements in feed efficiency by production of short-chain fatty acids [SCFA] (146, 152). Resistant starch increased the relative abundance of Lachnospiraceae-, *Prevotella*-, and *Rumicnoccus*- affiliated phylotypes in pigs, and resulted in increases in the production of SCFA (154). Furthermore, Belobrajdic et al. (155) demonstrated that a arabinoxylan-containing diet enhanced caecal fermentation and protected colonocyte DNA against diet-induced damage in pigs. But the main drawback of these kinds of *in vivo* research is that the diets are not controllable. Inevitably, extra carbon sources will be introduced to the formula, which does not allow to fully explain the result mechanistically. These mechanisms could be studied in *in vitro* models, that allow much more control over the intervention.

1.6 *In vitro* fermentation models

It is complicated and costly to investigate the effect of feed fermentation by the gut microbiota in pigs *in vivo*, due to the interaction with other feed-components (such as other fibers) in the swine feed, the ethical considerations (such as using pathogens, toxic compounds, and animal experiments themselves) and other uncontrollable factors (such as infectious disease or death during the experiment) which are important for reproducibility of results. *In vitro* fermentative models are considered excellent alternatives to study the effect on the composition and/or activity of the gut microbiota without ethical constraints. They allow the screening of a large number of substances such as dietary ingredients and drugs, and even allow the use of radioactive compounds or pathogens. There are several fermentation models mimicking the large intestine of human beings (156-159). Nowadays, two main types of *in vitro* fermentation models have been developed: batch fermentation models, and dynamic fermentation models.

1.6.1 Batch fermentation model

Barry et al. (159) described a simple *in vitro* batch system allowing estimation of the fermentability of dietary fiber in the *in vitro* model in five European laboratories. It is a one compartment fermentation model to mimic the human colonic environment. To perform fermentation experiments in the model, small reactor vessels or test-tubes are usually used, in which different cultures, such as specific strains, intestinal or fecal microbial communities from animal or human origin, are tested for their ability to metabolize different substrates. Relying on the one compartment system (bottles), one advantage of the system is that the anaerobic conditions can be easily maintained as the system is closed and air-tight. Another distinctive characteristic of one compartment *in vitro* colon models is the use of a buffer with minerals as the main matrix of the medium instead of nutritive medium with additional carbon sources. The main down side of these systems however is that they are not equipped with a dialysis system, and, as a result, the microbiota should be significantly affected by the accumulation of the end products of the microbial metabolism. Thus, pH control and long term fermentations are not possible. These simple system are defined as “static” cultures, which are far from physiological (160). Therefore, “dynamic” fermentation models have also been developed.

1.6.2 Dynamic fermentation model

The most sophisticated dynamic continuous single-stage fermentation model is represented by the multiple-compartment system TIM-2 (161), and two representative three-stage fermentation models are the Simulator of the Human Intestinal Microbial Ecosystem [SHIME] (157) and SIMulator Gastro-Intestinal [SIMGI] (158), which are technically upgraded from the three-stage culture reactor of Gibson and Macfarlane in the 1980s (162, 163).

1.6.2.1 TIM-2

The TNO *in vitro* model of the colon, nick-named TIM-2, is a model that closely mimics the physiological conditions of the human proximal large intestine (Figure 1)(164), in which all parameters are computer-controlled. In brief, TIM-2 consists of four connected glass-jackets with flexible silicon membranes inside. Peristaltic movements are used to mix and move chyme through the system, by applying pressure on the flexible walls. This mixing is better than the movement in batch fermentation models and stirrers in SHIME (discussed below), where fluids and solid phases may separate. A unique design of the system is that TIM-2 is equipped with a dialysate system, a dialysis membrane running through the model and a bottle collecting the spent dialysate. This prevents accumulation of microbial metabolites, which are normally absorbed by the gut lining, and would lead to the inhibition or death of the microbes in the model when they would accumulate. The dialysis system thus maintains a highly active microbiota with a similar density as that found in the human large intestine (165). In batch fermentation models or less sophisticated models the microbiota is usually inoculated at a 100-fold or even lower density, which leads to slow growth to physiological densities. As the microbial metabolites are also taken up by the epithelial cells of the colon *in vivo*, TIM-2 mimics better the physiological environment in the gut. Since all metabolites are collected, an approximately 100% mass-balance can be made, which is not possible *in vivo* in humans. This is not even possible in animals, despite that they may be sometimes euthanized in animal trials, since part or all of the metabolites can be metabolized by colonocytes and do not reach the blood circulation, or are quickly used by tissue before (blood) sampling takes place. Therefore, TIM-2 can be used to study (molecular) mechanisms. For example, stable-isotope (^{13}C) labeled carbohydrates were fed to TIM-2 and the degradation of these carbohydrates was followed over time (166, 167). After incubating in TIM-2, NMR and LC-MS were used to trace the fate of these labeled substrates. TIM-2 is also highly reproducible, this has been shown in numerous studies (168-170). Of course, *in vitro* models have their limitations. Similar to every other *in vitro* model that mimics the colon, TIM-2 has no epithelial cells or immune system, and lacks feed-back mechanisms.

Figure 1. TIM-2 schematic. a) peristaltic compartments with a hollow fiber membrane inside, b) pH sensor, c) NaOH secretion, d) dialysate system, e) level sensor, f) gaseous N₂ inlet, g) sampling port, h) gas outlet, i) test compound + feeding syringe, j) temperature sensor.

1.6.2.2 SHIME

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) is a multi-compartment dynamic simulator of the human gut (157). The conventional SHIME consists of a succession of five reactors simulating the entire gastrointestinal tract. The first two reactors, mimicking stomach and small intestine, follow the fill- and-draw principle adding a specific amount of nutritional medium, pancreatic enzymes and bile to the simulated stomach and small intestinal compartments. The remaining three compartments mimic the ascending, transverse, and descending colon, respectively (171). The colonic digestion phase is initiated by pumping the chyme of the small intestine compartment in the ascending colon vessel. Compared to TIM-2, SHIME has a few advantages. Firstly, it connects stomach and small intestine with colon together, while the TIM system consists of two separate machines (TIM-1 (156), mimicking stomach and small intestine; TIM-2 (161), mimicking the colon); secondly, recently, SHIME has been optimized to mimicking mucosal microbial colonization, and can also be coupled to a Host Microbiome Interaction module with epithelial or immune cells (172-174). However, the main drawback of SHIME is that no dialysis system is present, and as a result, SHIME cannot properly mimic the environmental conditions as discussed above. SHIME also has no feed-back mechanisms.

1.6.2.3 SIMGI

The SIMGI (SIMulator Gastro-Intestinal) is an automated gastrointestinal *in vitro* model, which, like SHIME, comprises five interconnected chambers that simulate the stomach, small intestine and three stages of colon (158). Since this system is quite similar to SHIME, we do not describe the model here. Details can be found in the literature (158). It looks like SHIME, but has some advantages taken from TIM-2. For example, its stomach uses peristaltic movements for mixing instead of stirring in the reactor, and the system is fully computer-controlled, which leads to more controllable pH and stomach emptying. No dialysis system, no feed-back mechanisms, and no host-microbe interactions are its limitations.

1.7 Aim and outline of the thesis

The aim of the thesis was to assess whether fermentation by swine gut microbiota of RSM,

which was processed by enzymatic or chemical treatment, was improved compared to untreated RSM, and whether the microbiota composition and activity changed. In addition, we believed that the feeding methods had significant effects on the efficiency of fibre utilization, and therefore, the effect of different feeding methods on RSM fermentation were explored in the thesis.

Chapter 2 describes the development of the Swine Large Intestinal *in vitro* Model (SLIM), which was developed based on the human, computer-controlled, dynamic TNO *in vitro* model of the colon (TIM-2). The medium used for the TIM-2 (SIEM, for simulated ileum efflux medium) was optimized for the pig-microbiota (SIEMP). And pig colonic pH (5.9) and temperature (39 °C) were implemented. In **Chapter 3**, the developed SLIM-system was used to study effects of RSM on the pig microbiota. RSM was modified by enzyme (a cellulase or 2 pectinases) or chemical (6 N alkaline) treatment. A shot of 5 g RSM (modified or not) were directly fed to the standardized swine gut microbiota after the system adaption period, and the effect of processing methods on the RSM utilization and microbiota composition was studied. Enzyme and chemical treatment as well as degradation by the pig microbiota of the fibre was studied using a suit of monoclonal antibodies directed against carbohydrates. In **Chapter 4**, the modified RSM was fed to SLIM continuously for 48 h, and investigated whether degradation of modified RSM was improved compared to untreated RSM and whether the microbiota composition was changed. In **Chapter 5**, the swine gut microbiota was adapted to modified RSM in SLIM for 48 h first, which was followed by a shot of 5 g modified RSM as evaluation period. Afterward, the degradability of modified RSM was validated *in vivo* (in cannulated pigs) by the mobile nylon bag technique. **Chapter 6** describes a preliminary novel high-throughput technique to screen polysaccharide structures, as a tool to further study fibre degradation by the (pig) gut microbiota. Results of all parts of the thesis are summarized and discussed in **Chapter 7**.

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Chapter 2

Polysaccharide source altered ecological network, functional profile, and short-chain fatty acid production in a porcine gut microbiota

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Abstract

Several validated dynamic *in vitro* models of the colon have been developed for humans, but there is no dynamic *in vitro* fermentation model for pigs. This study was conducted to modify the human, dynamic, computer-controlled TNO *in vitro* model of the colon (TIM-2) for pigs and investigate effects of different starch sources and polysaccharides on swine microbiota structure, ecological network, predictive functional profile, and short-chain fatty acids production. Our study showed that three different types of starch or two polysaccharides greatly impacted microbiota composition. Co-occurrence network analysis indicated that microbiota fed with different sources of starch changed the network topological properties. Functional profiles were predicted to vary significantly among the three starch treatments, and the original pig fecal inoculum was more similar to maize starch treatment. On the other hand, compared with maize starch and arabinoxylans (AX), the microbial composition of the original inoculum was more similar when AX-XG (arabinoxylans and xyloglucan) were added, and the functional profile of the original inoculum also clustered with AX-XG. The cumulative production of acetic, propionic, and butyric acid on maize starch were significant higher than those on potato starch and wheat starch, while only the amount of acetic acid was significant higher on AX-XG than that on AX. In conclusion, supplementation of maize starch as the starch source together with AX and XG, leads to the bacteria being more stable in the *in vitro* model and closer to the original inoculum and microbial function compared to potato starch, wheat starch and AX. A maize basal diet may improve energy absorption in the large intestine in growing pigs.

Introduction

Nowadays, it is well known that the gut microbiota plays a crucial role in the human and animal gut, which is related to the health status of the host and also to nutrient digestibility (1, 2). Pigs are both an important meat source for human consumption and an animal model for biomedical research. Thus, there is numerous research about pig gut microbiota (3-6). Ramayo-Caldas et al. (1) have studied the fecal microbiota from 518 healthy piglets, and reported that the dominant microbial phyla are Firmicutes and Bacteroidetes, which are in agreement with other reports (7-10). The remaining phyla contain Actinobacteria, Proteobacteria, and others (1, 7-9). The microbial abundances in lower phylogenetic levels are highly variable, but the bacterial types are quite similar. For instance, at family level, 11 families in pig gut microbiota are reported: *Prevotellaceae*, *Veillonellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Paraprevotellaceae*, S24-7 (identified as *Muribaculaceae*), *Campylobacteraceae*, *Clostridiaceae*, and others (3). A similar amount of family types are reported in another study (10), and some additional ones are *Streptococcaceae*, *Lactobacillaceae*, *Erysipelotrichaceae*, and *Coriobacteriaceae*. The same findings are observed at genus level, namely that the core genera reported by different researchers are similar, but the abundances are highly variable (3, 10, 11), e.g., due to different diet intake. Dietary fiber and resistant starch are known to modulate the intestinal microbiota and thereby contribute to gut health status by promoting the growth of beneficial microorganism in pigs and contribute to improvements in feed efficiency by production of short chain fatty acids [SCFA] (3, 10). Resistant starch increased the relative abundance of *Lachnospiraceae*-, *Prevotella*-, and *Ruminococcus*- affiliated phylotypes in pigs as a result of increases in the production of SCFA (10). Furthermore, Belobrajdic et al. (12) demonstrated that arabinoxylan-diet enhanced caecal fermentation and protected colonocyte DNA against diet-induced damage in pigs. But the main drawback of these kinds of *in vivo* research is that the diets are not controllable. Inevitably, extra carbon sources will be introduced to the formula, which does not allow to fully explain the result mechanistically.

It is complicated and costly to investigate the effect of feed fermentation by the gut microbiota in pigs *in vivo*, due to the interaction with other feed-components (such as other fibers) in the swine feed, the ethical considerations (such as using pathogens, toxic compounds, and animal experiments itself) and other uncontrollable factors (such as infectious disease or death during the experiment, which are important for reproducibility of results). *In vitro* fermentative models are considered excellent alternatives to study the effect on the composition and/or activity of the gut microbiota without ethical constraints. They allow the screening of a large number of substances such as dietary ingredients and drugs, and even allow the use of radioactive compounds or pathogens. There are several fermentation models mimicking the large intestine of human beings (13-16).

Nowadays, two main types of *in vitro* fermentation models have been developed: batch fermentation models, and dynamic fermentation models. Barry et al. (16) described a simple *in vitro* batch system, which is a one compartment fermentation model to mimic human colonic environment. The main side effect of these systems however is that they are not equipped with a dialysis system, and, as a result, the microbiota should be significantly affected by the accumulation of the end products of the microbial metabolism, such as SCFA. Thus, pH control and long term fermentations are not possible. These simple system are defined as “static” cultures, which are far from physiological conditions (17). Therefore, dynamic fermentation models have also been developed. The most sophisticated dynamic continuous single-stage fermentation model is represented by the multiple-compartment

system TIM-2 (18), and two representative three-stage fermentation models are the Simulator of the Human Intestinal Microbial Ecosystem [SHIME] (14) and SIMulator Gastro-Intestinal [SIMGI] (19), which are technically upgraded from the three-stage culture reactor of Gibson and Macfarlane in the 1980s (20, 21).

The TNO *in vitro* model of the colon, nick-named TIM-2, is a model that closely mimics the physiological conditions of the human proximal large intestine [Figure S1] (18), in which all parameters are computer-controlled (elaborated in the Materials and Methods section). In brief, TIM-2 consists of four connected glass-jackets with flexible silicon membranes inside. Peristaltic movements are used to mix and move chyme through the system, by applying pressure on the flexible walls. This mixing is better than the stirring in batch fermentation models or other *in vitro* models, where fluids and solid phases separate. A unique design of the system is that TIM-2 is equipped with a dialysate system, a dialysis membrane running through the model and a bottle collecting the spent dialysate (Figure S1). This prevents accumulation of microbial metabolites, which would lead to the inhibition or death of the microbes in the model when they would accumulate. The dialysis system thus maintains a highly active microbiota with a similar density as that found in the human large intestine (22). In batch fermentation models or less sophisticated models the microbiota is usually inoculated at a 100-fold or even lower density, which leads to slow growth to physiological densities. As the microbial metabolites are also taken up by the epithelial cells of the colon *in vivo*, TIM-2 mimics better the physiological environment in the gut. Since all metabolites are collected, an approximately 100% mass-balance can be made, which is not possible *in vivo*. This is not even possible in animals, despite that they may be sometimes euthanized in animal trials, since part or all of the metabolites can be metabolized by colonocytes and cannot reach the blood circulation, or are quickly used by tissue before sampling takes place. Therefore, TIM-2 can be used to study (molecular) mechanisms. For example, stable-isotope (^{13}C) labeled carbohydrates were fed to TIM-2 and the degradation of these carbohydrates was followed over time (23, 24). After incubating in TIM-2, NMR and LC-MS were used to trace the fate of these labeled substrates. TIM-2 is also highly reproducible, this has been shown in numerous studies (5, 25, 26). Of course, *in vitro* models have their limitations. Similar to every other *in vitro* model that mimics the colon, TIM-2 has no epithelial cells or immune system, and lacks feed-back mechanism.

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) and SIMGI (SIMulator Gastro-Intestinal) are multi-compartment dynamic simulators of the human gut (14, 15). Compared to TIM-2, SHIME and SIMGI have their own advantages. For instance, they connect stomach and small intestine with colon together, while the TIM system consists of two separate machines [TIM-1 (13), mimicking stomach and small intestine; TIM-2 (18), mimicking the colon]. However, the main drawback of SHIME and SIMGI is that no dialysis system is present, and as a result, they cannot properly mimic the environmental conditions as discussed above. They also have no feed-back mechanism.

Mostly, these systems have been used to study the human microbiota. To our knowledge, advanced and dynamic *in vitro* models for swine studies have not been developed, and TIM-2 has its own unique advantages compared with other *in vitro* models. Therefore in this study, the authors intended on changing the parameters of TIM-2, which is mimicking the colonic physiological conditions of human beings, to the parameters of the swine large intestine, such as pH, temperature, and the growth medium. The growth medium for the gut microbiota was initially developed by (20) which was subsequently used for TIM-2 (standard ileum effluent medium, SIEM), SHIME, SIMGI, and other models in the field with minor modifications.

The medium comprises a source of protein and complex carbohydrates (i.e. resistant starch, pectin, arabinogalactan, xylan) that are not digested by the human enzymes. In the current study, the medium was optimized for use by the swine gut microbiota, as the strategy of modifying TIM-2 to swine *in vitro* large intestinal model (named SLIM). Based on this, two studies were designed: **Study I**, to assess the effect of different starch sources (potato, wheat, and maize starch) on pig microbiota in SLIM (in order to define the optimized starch source to supplement to standard ileum effluent medium for pigs, SIEMP); **Study II**, after selecting the optimal starch in the SIEM recipe, two more polysaccharides (xyloglucan and arabinoxylan) were added to the recipe of SIEM to investigate their effect on pig microbiota (to optimize SIEMP). Study I was trying to clarify which starch source fits pig microbiota *in vitro* best. Study II was designed to best mimic the polysaccharide composition of swine basal diets to allow growth of most of the members of the pig microbiota.

1 Materials and Methods

1.1 The Swine *in vitro* large intestinal model

The Swine *in vitro* Large Intestinal Model (SLIM) (Figure S1) was established on the basis of the human, computer-controlled, dynamic TNO *in vitro* model of the colon, TIM-2 (18), which simulates the physiological conditions in the lumen of the human proximal colon. In brief, the units, consisting of 4 interlinked glass compartments with a flexible membrane inside (Figure S1a), were flushed with nitrogen (Figure S1f) prior to the introduction of the inoculum, and throughout the remainder of the experiment. The volume of the lumen is 120 mL.

The system was maintained under the condition of porcine body temperature (39 °C) using a temperature sensor (Figure S1j; Easytem R31, Endress + Hauser, Nesselwang, Germany) for 96 h with the pH kept at or above 5.9 by automatic titration with 2 M NaOH (Figure S1c). In order to prevent the accumulation of microbial metabolites, which would otherwise inhibit and/or kill the microbiota in a matter of 5-6 hours, water and microbial metabolites were removed from the lumen using a dialysate system (Figure S1d; described below) consisting of a semi-permeable hollow membrane which runs through the lumen. For all the experiments, the speed of the dialysis fluid was set at 1.5 ml min⁻¹ (27). Mixing and transport of the intestinal contents occurs through the simulation of peristaltic movements, by squeezing of the flexible membrane. The system was inoculated with a standardized, complex, high density, metabolically active, anaerobic microbiota of swine origin.

1.2 Feces collection and standardization

Grab samples, fresh and not contaminated with urine, were collected from the floor from growing pigs (48 pens with 6 pigs/pen, Hypor Libra x Hypor Maxter, Hendrix Genetics, Boxmeer, the Netherlands), and were put into a bag and placed immediately into boxes with gastight lids, containing an Anaerocult® strip (AnaeroGen™, Cambridge, UK) to create anaerobic conditions during transport to the anaerobic cabinet. This transport never lasted longer than 3 hours. The pigs received no antibiotics and their body weight was approximately 70 kg. Pigs were fed *ad libitum* with a commercial diet, which was formulated to meet or exceed the nutrient requirements of growing pigs according to CVB (Dutch research institute for livestock feed and nutrition). Since this was not an intervention study, feces collection from the floor of the pens of the pigs did not require ethical approval in accordance with local/national guidelines.

The standardized pig microbiota used to inoculate in the SLIM system was prepared in an anaerobic cabinet [Sheldon Lab – Bactron IV, Gompelius, OR, USA] (22). The fecal samples were weighed and mixed with an equal weight of dialysate solution (described below). Then, glycerol was added to final concentration of 15% and the slurry was mixed well. The fecal material was aliquoted (45ml) in 50 ml tubes, snap-frozen in liquid nitrogen and stored at -80 °C.

1.3 Study I: The effect of different starch sources on pig microbiota

1.3.1 Experimental setup

To start the experiment, SLIM was inoculated with 60 ml of the standardized microbiota (described above) plus 60 ml of dialysis liquid (described below) yielding a total of 120 ml (total volume of the system). The microbiota was fed with simulated ileal effluent medium (described below), but the original starch source as described by (18) was replaced by potato (4.5 g d⁻¹), wheat (4.5 g d⁻¹), or maize starch (4.5 g d⁻¹).

1.3.2 Simulated ileal effluent medium and dialysate

The simulated ileal effluent medium (SIEM), slightly modified from (20) and described in

(27) together with the dialysate composition, contained the following components (g L^{-1}): 74.6 starch (potato, wheat or maize), 9.0 xylan, 9.0 pectin, 9.0 amylopectin, 9.0 arabinogalactan, 31.5 Tween 80, 43.7 casein, 0.7 ox-bile, 43.7 bactopecton, 4.7 $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.009 $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8.4 NaCl, 0.8 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.7 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 bile, 0.02 haemin and 0.3 cysteine-HCl, plus 1.5 mL of a vitamin mixture containing (per litre): 1 mg menadione, 0.5 mg vitamin B12, 2 mg D-biotin, 10 mg pantothenate, 5 mg p-aminobenzoic, 4 mg thiamine and 5 mg nicotinamide acid. The pH was adjusted to 5.9. Dialysis liquid contained (per litre): 2.5 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.005 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 g NaCl, 0.45 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g bile, 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.4 g cysteine-HCl, plus 1 mL of the vitamin mixture. All medium components were purchased at Tritium Microbiology (Eindhoven, The Netherlands). SIEM only contains indigestible carbohydrates and hence did not require pre-digestion. SIEM was administered at 60 ml d^{-1} [corresponding to $7.5 \text{ g carbohydrate d}^{-1}$] (28).

1.4 Study II: The effect of polysaccharides on pig microbiota

Except for SIEM all other procedures were the same as for the above experiment. Starch of SIEM was changed to the optimal source discovered in the above experiment (maize). Moreover, arabinoxylan (AX, 0.54 g d^{-1} ; BioActor, Maastricht, the Netherlands), or arabinoxylan (0.54 g d^{-1}) and xyloglucan (0.54 g d^{-1} ; CarboMer, Inc., San Diego, CA USA) together (AX-XG) were added to the SIEM recipe.

1.5 Sample collection and statistics

Four SLIM-units were run in parallel each time. Experiments were performed in duplicate ($n=2$). Each starch source (Study I) or non-starch polysaccharides (Study II) was run in two units, and samples of each unit were taken from the lumen and dialysate after 24, 48 and 72 hours to analyze microbiota composition and SCFA production. Samples were snap-frozen in liquid nitrogen and stored until analyses (described below). After 24 and 48 h of fermentation 25 ml of lumen sample was removed from the system to mimic the transit of chyme from the proximal to the distal colon (27).

1.5.1 Microbial DNA extraction and sequencing of the V3-V4 region of the 16S rRNA gene

Genomic DNA extraction from a single sample at each time point was performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research) according to the manufacturer's instructions.

Illumina 16S rRNA gene amplicon libraries were generated and sequenced by BaseClear (Leiden, the Netherlands). In short, barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step PCR. 10-25 ng genomic DNA was used as template for the first PCR with a total volume of $50 \mu\text{l}$ using the 341F ($5'\text{-CCTACGGGNGGCWGCAG-3'}$) and the 785R ($5'\text{-GACTACHVGGGTATCTAATCC-3'}$) primers (29) appended with Illumina adaptor sequences. PCR products were purified (QIAquick PCR Purification Kit) and the size of the PCR products were checked on a Fragment analyzer (Advanced Analytical, Ankeny, US) and quantified by fluorometric analysis (Qubit™ dsDNA HS Assay Kit). Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes.

The raw sequencing data produced was processed using tools available in QIIME 2 (Version

2017.11) (<https://qiime2.org>). Reads were imported and quality filtered and dereplicated with q2-data2 (30). Dada2 was performed with paired-end reads. Truncation parameters were as follows: for forward reads the first 17 base pairs were trimmed off, and truncated at position 280 base pairs; for reverse reads the first 21 base pairs were trimmed off, but these were truncated at position 230 base pairs. The resulting data was used for further analysis. The q2-phylogeny plugin (<https://github.com/qiime2/q2-phylogeny>) was used to generate a tree for phylogenetic diversity analyses. Alpha-diversity (Shannon, Evenness and Faith's PD) and beta-diversity analyses were performed with the q2-diversity plugin (<https://github.com/qiime2/q2-diversity>). At study I, study II, and study III, in order to include all the samples, we selected 15177, 17638, and 9227 as sampling-depth, respectively. Inverse Simpson was calculated by the package vegan in R. Shannon, Faith's PD, and inverse Simpson were compared among starch sources (or non-starch polysaccharides sources) and time points by a Two-way-ANOVA by the R function aov() in the built-in R package, and multiple pairwise-comparison between the means of groups was performed by Tukey HSD (Tukey Honest Significant Differences, R function: TukeyHSD()) in the built-in R package. The results were visualized by ggpubr R package. Distance matrices were calculated by unweighted (qualitative) and weighted (quantitative) UniFrac to compare the microbiota of individual units used in the study, where the former only considers their presence or absence, whereas the latter accounts also for abundance of observed organisms (31). In QIIME2 the script "qiime diversity beta-group-significance --i-distance-matrix --m-metadata-column --p-method --p-pairwise --p-permutations --o-visualization" was used for significance testing on distance matrices. The script performs PERMANOVA analyses (32). Factors of starch sources and non-starch polysaccharides were tested with PERMANOVA. Taxonomic analysis was done using the q2-feature-classifier plugin (33). The classifier was trained on Silva_128_release using the 99% identity level for amplicon sequence variant (ASV), where the sequences had been trimmed to only include 400 bases from the region of the 16S that was sequenced in this analysis (the V3-V4 region, bound by the Bakt_341F/ Bakt_785R primer pair). Networks were constructed by Conet (34), analyzed by NetworkAnalyzer (35) and visualized in Cytoscape (36). For constructing co-occurrence networks, the feature (ASV) table was split into three datasets: maize starch, potato starch, and wheat starch. For each of the datasets, only the ASV that appeared in 3 or more in the total of 6 samples were kept for downstream analysis. Five methods (Pearson, Spearman, Mutual Information, Bray Curtis and Kullback-Leibler dissimilarity) were selected for the ensemble inference. MCODE (37), a Cytoscape plugin, was used to uncover modules in the three networks. Linear discriminant analysis effect size [LEfSe] (38) was used to find biomarkers between groups using relative abundances from the feature tables generated in QIIME. The feature tables were filtered as follows: only ASV with a mean of all the samples greater than 10^{-5} were kept; taxa not seen more than 3 times in at least 20% of the samples were removed before LEfSe analysis. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States [PICRUSt] (39) was used to predict the gene families contributing to the metagenomes of the samples identified using 16S rRNA sequencing (KEGG database was used to predict the results). This data was visualized using statistical analysis of taxonomic and functional profiles (STAMP) (40). Raw sequencing data was submitted to the European Nucleotide Archive under the accession number: PRJEB31280.

1.5.2 SCFA (acetate, propionate and butyrate) analysis

Samples for determination of concentrations of SCFA were analyzed by Brightlabs (Venlo, The Netherlands). Ion exclusion chromatography (IEC) was applied on an 883 Ion

Chromatograph (IC; Metrohm, Switzerland), using a Transgenomic IC Sep ICE-ION-300 column (30 cm length, 7.8 mm diameter and 7 μm particles) and a MetroSep RP2 Guard. The mobile phase consists of 1.5 mM aqueous sulphuric acid. A column flow rate of 0.4 ml min^{-1} was used. The temperature of the column was 65 $^{\circ}\text{C}$. The organic acids were detected using suppressed conductivity detecting. Samples were centrifuged at 14,000 rpm (K243R Refrigerated Centrifuge, Pro-Research, UK) for 10 minutes, and the clear supernatant was filtered through a 0.45 μm PFTE filter and diluted with mobile phase (for lumen 1:5, for dialysate 1:2). Ten microliters were loaded on the column by an autosampler 730 (Metrohm). Molecules were eluted according to their pKa. Significance of SCFA concentrations among different treatments was analyzed by ANCOVA with the built-in R function aov() (version 3.5.2), and bar charts were made in Microsoft Office.

2 RESULTS

2.1 The Effect of Different Starch Sources on Pig Microbiota

2.1.1 Microbiota diversity

Alpha-diversity analyses were performed on the samples from each unit of the *in vitro* model over the 72 hour period and showed that there was no significant difference in Shannon index among the different diets. However, Faith's PD and inverse Simpson indices were significantly changed among the different diets (Figure S2). The Faith's PD index of the microbiota treated with maize starch was significantly higher than that of microbiotas treated with potato or wheat starch. The inverse Simpson index of the microbiota fed with wheat starch was considerably higher than that fed with maize or potato starch (Figure S2). Shannon, Faith's PD, and inverse Simpson indices of the microbiotas treated with the three different starches decreased over time, where the wheat starch fed group fell at the fastest rate, followed by potato starch and maize starch fed groups, respectively (Figure S2). All indices were significantly lower at time point 72 hours, compared to time point 24 hours (Figure S2). Figure 1 demonstrates that pig microbiota communities were clustered according to the substrate that was fed (unweighted: $P = 0.001$, weighted, $P = 0.001$) (Figure 1). The dissimilarity in β -diversity within the same time points of wheat starch fed microbiota assessed with weighted UniFrac was bigger than that with unweighted UniFrac, which demonstrated that ASV abundance affected the dissimilarity of the wheat starch fed microbiota. A PERMANOVA analysis of the Bray-Curtis distances showed a similar results, where Bray-Curtis metrics were significantly different among the three starch sources (data not shown). After feeding maize, potato, and wheat starch, the original inoculum sample separated with samples from maize, potato, and wheat starch.

Figure 1: Principal coordinate analysis (PCoA) plots generated based on the calculated distances in a weighted (A) and unweighted (B) UniFrac matrix. Samples were grouped by color (time-points) and shape (in terms of diet group) they belonged to. The ellipses were drawn at the 0.95 confidence level. Diet sources and original were significant different from each other, for weighted uniFrac: Original inoculum to Maize starch ($P=0.041$), to Potato starch ($P=0.031$), to Wheat starch ($P=0.033$); Maize starch to Potato starch ($P=0.002$), to Wheat starch ($P=0.002$); for unweighted uniFrac: Original inoculum to Maize starch ($P=0.046$), to Potato starch ($P=0.041$), to Wheat starch ($P=0.048$); Maize starch to Potato starch ($P=0.022$), to Wheat starch ($P=0.006$).

2.1.2 Taxonomic affiliations

The dominant bacterial phyla were Firmicutes (48%, 46%, 48%, and 50% in maize starch, potato starch, wheat starch and original inoculum experiments, respectively), Bacteroidetes (17%, 27%, 24%, and 34% respectively), and Actinobacteria (33%, 17%, 12%, and 11% respectively) (Figure 2A, Figure S3). Figure 2B and Figure S3 shows the 14 most abundant families of the original inoculum and the three experimental groups. The relative abundance of the Prevotellaceae family in the original inoculum was much higher than those in maize,

potato, and wheat starch groups. At both phylum and family level, the microbial relative abundances between duplicates were quite similar and the bacterial abundances among the three different time points were also stable (Figure S3).

Figure 2: Relative abundances of microbial phyla (A) and families (B) in the original inoculum, and microbiome fed with different starches at different time points (24 h, 48 h, and 72 h).

Within the phylum Firmicutes, the families Lactobacillaceae, Streptococcaceae, Christensenellaceae, Clostridiales, Family XIII, and Erysipelotrichaceae were more abundant in maize starch treated microbiota compared to potato and wheat starch, while Acidaminococcaceae and Veillonellaceae showed higher abundance upon wheat starch addition compared to the other two starches (Figure 3). Among Bacteroidetes, Prevotellaceae was more abundant upon potato starch feeding compared to the other two starch substrates. Among Actinobacteria, Coriobacteriaceae was more abundant for maize starch, while Bifidobacteriaceae was more abundant for wheat starch (Figure 3). Within Proteobacteria, Alcaligenaceae, Succinivibrionaceae, and Enterobacteriaceae were more abundant for wheat compared to maize and potato starch (Figure 3). At the genus level (Figure S4), the relative abundance of *Coriobacteria*, *Olsenella*, *Holdemanella*, *Acidaminococcus*, *Mogibacterium*,

Solobacterium, *Lactobacillus*, *Coprococcus3*, *Streptococcus*, *Dorea*, and *Catenibacterium* were significantly higher for maize starch, while the relative abundance of *Prevotella9*, *Acetitomaculum*, *Ruminococcus2*, *Coprococcus1*, *Syntrophococcus*, *Sharpea*, and *Mitsuokella* were significantly higher for potato starch group. The relative abundance of *Succinivibrio*, *Negativicutes*, *Megasphaera*, *Bifidobacteriales*, *Actinobacteria*, *Citrobacter*, and *Sutterella* were significantly higher for wheat starch.

Figure 3. LefSe results on starch-fed pig microbiota showing taxonomic representation of statistically and biologically consistent differences among maize, potato, and wheat starch treated microbiota. Differences are represented by the color of the substrates (red, green, and blue indicating Maize, Potato, and Wheat starch, respectively).

2.1.3 Ecological network

As shown in Figure S5 and Table 1, the topological structures were different among maize, potato, and wheat starch. There were 325 nodes connected by 3349 edges, 239 nodes connected by 2874 edges, and 264 nodes connected by 3194 edges in maize, potato, and wheat starch-based networks, respectively. This indicates that maize starch-based network was larger. The R^2 fitting of the three networks was 0.709 (maize starch), 0.543 (potato starch), and 0.536 (wheat starch), respectively. This suggests that the scale-free property of the maize starch-based network was better than those of the networks for potato and wheat starch.

To detect dense modules and to explore the biological patterns in the three starch networks, the tool MCODE (37), a plugin for Cytoscape, was applied to analyze the networks. We detected 14 modules, 8 modules, and 15 modules present in maize, potato, and wheat starch networks, respectively. Figure 4 shows the top three highly-connected modules present in

each of the three starch networks. In the largest module (module 1), maize starch contained 30 nodes connected by 624 edges. Among them, 20 nodes belonged to the phylum Firmicutes, and the rest nodes belonged to Bacteroidetes (6 nodes), Actinobacteria (3 nodes), and Proteobacteria (1 node). Module 1 of Potato starch contained 32 nodes and 565 edges, among which 16 nodes and 11 nodes belonging to Firmicutes and Bacteroidetes, respectively. The other 5 nodes were shared by the phyla Actinobacteria, Proteobacteria, and Euryarchaeota. With respect to wheat starch, the sub-network (module 1), contained 35 nodes and 803 edges, among which both Bacteroidetes and Firmicutes had 14 nodes, and Proteobacteria and Actinobacteria had 5 nodes and 2 nodes, respectively.

Figure 4: Top three highly-connected clusters in networks of porcine gut microbiota fed with maize, potato, and wheat starch. Node colors represent the different phyla. Green edges represent positive interactions, while red edges indicate negative interactions, and black ones are unknown.

To reveal the hub nodes of each network, the degree (number of edges connected to a node), positive degree (number of positive edges connected to a node), and negative degrees (number of negative edges connected to a node) were assigned to each node by Conet (Table S1) (34). The top connected nodes of maize, potato, and wheat starch were *Tuicibacter*, *Subdoligranulum*, and *Prevotella*, respectively, which were also their top positive hubs. The

top negative hubs belong to Megasphaera, Ruminococcaceae UCG-014, and Prevotellaceae, respectively.

2.1.4 Predictive functional profile

Given the structural changes within the bacterial community among maize, potato, and wheat starch treatment, we subsequently tested whether different starches caused functional profile changes within each microbiota too, and we compared this to an original pig fecal sample to reveal the functional similarity between different starch groups. PICRUSt (39) was applied to the 16S rRNA gene data to predict functional content of the metagenome. Twenty-eight active features were found to be significantly different among the three starch groups (Table S2). More specifically, in the top five most abundant differential features, the relative abundance of Membrane Transport for maize starch was significantly higher than the other two groups (maize starch : wheat starch, $P < 0.001$; maize starch : potato starch, $P < 0.01$). Moreover, for potato starch this was also significantly higher than for wheat (potato starch : wheat starch, $P < 0.05$). The relative abundance of Replication and Repair for maize starch was significantly lower (wheat starch : maize starch $P < 0.001$; potato starch : maize starch, $P < 0.001$) (Table S2). The relative abundance of Carbohydrate Metabolism for maize starch was significantly higher than the other two starches (maize starch : wheat starch, $P < 0.01$; potato starch : wheat starch, $P < 0.01$; maize starch: potato starch, $P < 0.01$), and that of wheat starch lower than that of potato starch. The relative abundance of Amino Acid Metabolism for wheat starch was significantly higher (wheat starch : potato starch, $P < 0.001$; wheat starch : maize starch, $P < 0.001$). The relative abundance of Translation for potato starch was significantly higher than that for wheat starch ($P < 0.05$) (Table S2).

The relative abundance of microbial functional profiles of the three starch groups and the original pig feces were used to create a PCA plot (Figure 5A) and heatmap (Figure 5B). Hierarchical clustering showed that samples from the same starch tended to cluster together, and the original pig microbiota clustered with the maize starch treated microbiota. The same result was observed in the PCA plot: samples from different starch treatments clearly separated, and the sample from the pig feces clustered with samples from maize starch (Figure 5A).

Figure 5. PCA (A) and heatmap (B) plot of microbial functional profile of maize (red), potato (green), and wheat starch (blue) treatments and the original pig fecal sample (orange).

2.1.5 Microbiota activity

Figure 6 shows the cumulative production of beneficial microbial metabolites SCFA (acetate, propionate and butyrate) after 72 hours of maize, potato, and wheat starch treatment. The cumulative amounts of acetic ($P = 0.006$), propionic ($P = 0.008$), and butyric acid ($P = 0.034$) produced upon maize starch feeding were significantly higher than for potato starch and wheat starch. This corroborated the predicted functional data that the relative abundance of Carbohydrate Metabolism for maize starch was significantly higher than that of the other two groups. SCFA production did not differ between wheat and potato starch.

Figure 6. Effect of different starches on the SCFA after 72 hours fermentation

2.2 Study II: The Effect of Different Non-Starch Polysaccharides on Pig Microbiota

Study I proved that predictive functional profile composition for maize starch treatment was most similar to that of the original pig inoculum. Therefore, maize starch was chosen as starch source in the SIEM recipe in study II. SIEM is optimized for humans. But, as the diet of human beings is different from pigs, the composition of other polysaccharides (besides starch source) should also be different. Moreover, pigs may obtain more fibers from their diet. Consequently, two more polysaccharides, which are present in maize or soybean meal, were added into the recipe of SIEM (arabinoxylans, AX; or arabinoxylans plus xyloglucan, AX-XG) to investigate their effect on pig microbiota functionality. The microbiome functional profile data of maize group of study I and the original pig feces was used to compare their differences among AX, and AX-XG group.

2.2.1 Microbiota diversity

There was no significant differences in Shannon index and Faith's PD between AX and AX-XG feeding (Figure S6), but inverse Simpson of microbiota fed with AX-XG was significantly higher than that fed with AX. Shannon index and inverse Simpson of AX-XG were slightly higher than those of maize starch, but they were not significant (data not shown). Additionally, Shannon index, Faith's PD, and inverse Simpson of time point 24 hours were significantly higher than those of time point 48 and 72 hours.

The added polysaccharides significantly affected beta-diversity (Figure S7). The weighted and unweighted UniFrac were used to calculate the phylogenetic distance matrix, and a PERMANOVA test was performed in each case to determine if the separation of sample groups was significant. The PERMANOVA test showed microbial profiles of AX treatment separated significantly from that of AX-XG treatment (weighted: $P = 0.003$, unweighted: $P = 0.034$).

Beta-diversity of maize starch from study I was introduced to compare with AX and AX-XG. There were significant differences between maize starch and AX-XG in beta-diversity (weighted: $P = 0.003$, unweighted: $P = 0.024$). The difference in unweighted UniFrac between maize starch and AX was not significant ($P = 0.141$), while it was significant for weighted UniFrac ($P = 0.003$). Figure S7 shows that difference among samples in AX-XG was smaller than those in AX, which means AX-XG had less individual difference and highly reproducibility between replicates.

2.2.2 Taxonomic affiliations

Stacked relative abundance bar plots were produced for all samples at phylum and family level (Figure 7 and Figure S8). Compared to the microbiota fed with maize starch (containing 17% Bacteroidetes, 33% Actinobacteria), the relative abundance of Bacteroidetes was increased in the microbiota fed with AX (28%), and AX-XG (30%), and the relative abundance of Actinobacteria was decreased for AX (22%), and AX-XG (19%), which made the microbial composition closer to the original inoculum (34% Bacteroidetes, 10% Actinobacteria). At phylum level, the microbial composition of AX-XG was more similar to the original inoculum than that of AX. Similar results are also shown at the family level. After feeding with AX and AX-XG, relative abundances of the families Prevotellaceae, and Succinivibrionaceae were increased and that of family Atopobiaceae was decreased, compared to the microbiota fed with maize starch, which made the microbiota composition more similar to the original inoculum. But all treatments failed to maintain similar levels of the families Lactobacillaceae and Christensenellaceae, which had higher relative abundance in the original inoculum (Figure 7). The microbial abundances (at phylum and family level) between duplicates were quite similar (Figure 7), and were stable among different time points, except for family Ruminococcaceae. During the first 24 hours of incubation, the relative abundance of Ruminococcaceae was quite similar to the original inoculum, but it dropped down at 48 hours and 72 hours.

Figure 7. Relative abundances of microbial phyla and families in the original inoculum, and microbiome fed with different substrates at different time points (24 h, 48 h, and 72 h).

At the genus level, the core genera in the original inoculum, and the microbiotas fed with different polysaccharides are shown in a PCA plot (Figure 8A), showing that the genus composition of the original inoculum was closer to AX-XG, compared to maize starch and AX. And samples in maize starch and AX also had larger individual differences than samples in AX-XG.

Figure 8. PCA (A) plot of microbial relative abundance at genus level of maize (red), AX (blue), and AX-XG (yellow) treatments and the original pig fecal sample (orange); Heatmap plot (B) of microbial functional profile of maize (red), AX (blue), and AX-XG (yellow) treatments and the original pig fecal sample (orange).

Most of the significant taxa affected by the treatment were observed in the AX-XG group (Figure S9). At the family level, four families were more abundant upon AX-XG treatment than after AX feeding: Methanobacteriaceae, Enterococcaceae, Streptococcaceae, and Acidiminococcaceae. In contrast, only one family (Bacillaceae) was more abundant for AX than for AX-XG. At the genus level, *Prevotella*, *Lactococcus*, *Succiniclasicum*, *Enterobacter*, and *Escherichia_Shigella*, were more abundant for AX-XG, while *Bacillus* had a significantly lower abundance compared to AX. There were no significantly difference at genus level between AX-XG and original inoculum, excepting for [*Ruminococcus*] *gauvreauii* group and *Dialister* (Table S7).

2.2.3 Predictive functional profile

Metagenome function was predicted using PICRUSt to investigate whether the diets would change the microbiome functional profile. Despite some differences in beta-diversity, only one significant feature was found, Immune system, of which the relative abundance was significantly higher ($P = 0.034$) for AX. Subsequently, the predicted microbiome functional profiles of the maize starch treatment from Study I and the original pig fecal sample were compared to that of AX and AX-XG group (Figure 8B). The heatmap plot (Figure 8B) shows that the relative abundances of the predictive functional metagenome of the original inoculum were similar to AX and AX-XG, but it clustered more with AX-XG.

2.2.4 Microbiota activity

Table S3 shows that there were no significant differences between AX and AX-XG with respect to the cumulative production of the beneficial metabolites propionate and butyrate after 72 hours fermentation. Acetate production was comparable for the two substrates, yet the amount of Acetate for AX-XG was significantly higher than that for AX ($P < 0.001$).

2.2.5 Reproducibility of microbiota fed with AX-XG

As described above, AX-XG was the optimized medium for SLIM, thus its microbial reproducibility was assessed by its replicates of alpha diversity, beta diversity, microbial

abundance, and microbial activities. The coefficients of variation (C.V.) of Shannon index and Faith's PD at time points 24, 48, and 72 h were all lower than 6 % (Table S4). Most of the C.V. of microbial abundance (at phylum and family level) were lower than 15 %, only 5 taxa, which represented 9.8 % of total taxa, were higher than 15% (Table S5). And most of them were lower than 10%. C.V. of acetic acid, propionic acid, and butyric acid were all lower than 14%, and most of them were lower than 5 % (Table S6).

3 Discussion

The TNO computer-controlled dynamic *in vitro* model of the colon (nick-named TIM-2) was developed by TNO approximately 20 years ago (18), and has been used extensively to study the effect of all kinds of dietary substrates on the human gut microbiota. For instance, the effect of probiotics and antibiotics on the intestinal homeostasis (41), the effect of numerous carbohydrates (42, 43), or fermentation of polyphenols from citrus, tea, or soy flavonoids into phenolic compounds with a more simple structure (44). The model is highly reproducible, as it is completely computer-controlled. Here, in order to investigate the pig gut microbiota *in vitro*, we modified the *in vitro* system to the swine colon (SLIM). As TIM-2 is originally designed for human beings, the colon parameters, such as temperature (37 °C), pH (5.8), and Standard Ileal Efflux Medium (SIEM), are adapted to the physiology of man. For SLIM, therefore temperature (39 °C), pH (5.9) and medium composition were modified to correspond to swine physiology (45, 46). Next generation sequencing was used to evaluate the effect of different starch sources and polysaccharides added to the SIEM medium on the microbiota of finishing pigs in the *in vitro* model during 72-hour fermentation experiments to investigate the optimal medium composition for pig gut microbiota. This study showed that starches from three different sources had significantly different effects on microbiota composition of finishing pigs and their predicted functional profiles. The microbial composition and predicted functional profile of original inoculum was similar to those of the microbiota fed with AX-XG, compared with the microbiota fed with maize starch and AX. Moreover, pig microbiota composition and activity fed with SIEMP (comprising of maize starch, AX, XG, and the rest of the compounds in normal (human) SIEM) were highly reproducible.

3.1 The Effect of Different Starch Sources on Pig Microbiota

Normally carbohydrates fermented by bacterial community in the colon belong to non-starch polysaccharides, but research showed that in humans 8-40 g starch per day may reach the colon (47, 48), which is termed resistant starch (49). In pigs, there is already substantial fermentation in the distal small intestine, and thus the amount of resistant starch that may reach the cecum/colon in pigs may differ, but we were unable to find any data on this. Resistant starch can be classified into four types, which are RS1 (physically inaccessible), RS2 (resistant granules), RS3 (retrograded starch), and RS4 (chemically modified). Feed processing, like heat, moisture, and pelleting, will affect the starch availability and formation of different types of RS (50). This RS reaching the swine colon will affect the microbiota composition and host health. In this *in vitro* study, SIEM (containing potato starch) was heated and mixed with other feed compounds during the preparation, and it contains RS. But the SIEM medium was developed to mimic a human diet. Thus, we aimed to investigate how the pig microbiota changes after feeding different sources of (resistant) starch.

Starch sources impacted the microbiota composition. Different starch sources had a considerable effect on both alpha-diversity (inverse Simpson, and Faith's PD), and beta-diversity (weighted and unweighted UniFrac, and Bray-Curtis) (Figure S2, Figure 1), indicating that some microbial species (composition and/or abundance) among the three

starch treatments were significantly different. Previous studies showed that certain starches can impact gut microbiota composition (4, 51). (4) demonstrated that microbiota fed with starches with the same RS type clustered together, while starches with different RS types significantly separated (4). (52) showed retrograded potato starch (RS3), but not native potato starch (RS2), promotes fecal and cecal *Lactobacillus* counts in rats. Another study showed that a chemically modified RS (RS4) increased *Bifidobacterium adolescentis* and *Parabacteroides distasonis*, while a RS2 led to significant increases in the proportions of *Ruminococcus bromii* and *Eubacterium rectale* when compared to RS4 (53). In the current study, among the three starch treatments, 11 genera, 7 genera and 7 genera were more abundant upon maize, potato, and wheat starch treatment, respectively (Figure 3, Figure S4). The inherent properties of starches, such as crystallinity, granular structure, amylose:amylopectin ratio, and retrogradation of amylose, will influence the formation of resistant starch (54). Therefore, the proportion and amount of RS types of starch sources may lead to alteration of the microbiota composition after feeding these starches.

The ensemble method was used based on multiple similarity measures in combination with generalized boosted linear models on microbial abundance (55). This investigates whether the shift of microbial abundance will change the bacterial co-occurrence relationships. Our results showed that maize starch-based network had much better scale-free properties, as evaluated by the value of degree distribution fitting R^2 (Table 1). Scale-free networks display a very strong robustness against random failures (56). Thus microbiota fed with maize starch may be more stable or resilient, compared to those of potato and wheat starch.

Table 1 Topological properties of networks of porcine gut microbiota fed with different sources of starch.

Network property	Maize starch	Potato starch	Wheat starch
Number of nodes	325	239	264
Number of edges	3349	2874	3194
Clustering coefficient	0.361	0.389	0.476
Network diameter	10	8	8
Characteristic path length	3.369	3.188	3.127
Network density	0.041	0.069	0.058
Network heterogeneity	1.088	1.027	1.005
Multi-edge node pairs	778	628	756
Degree distribution fitting slope	-0.807	-0.617	-0.626
Degree distribution fitting R^2	0.709	0.543	0.536

Networks of microbiota fed with different sources of starch had different keystone species (Figure 4, Table S1). The top ten highly-connected nodes in the maize starch-based network were all different from those of potato and wheat starch-based network. Moreover, Figure 4 shows that the top three highly-connected clusters of the maize starch had more hub nodes from the phylum Firmicutes, while potato starch had more hub nodes from the phylum Bacteroidetes. Wheat starch contained more hubs from the phylum Proteobacteria, compared to maize and potato starch. A high ratio of Firmicutes:Bacteroidetes are characterized as a trait of obesity, which goes hand-in-hand with observations of high levels of SCFA in the gut (57-59). In our study, hub nodes of the maize starch-based network showed that it was dominated by Firmicutes (Figure 4). Likely as a consequence of this, SCFA production of maize starch fed microbiota was significantly higher than that of potato and wheat starch ones (Figure 7), and the corresponding (predicted) microbial function was also changed. Metagenome prediction revealed that gene functions associated with Carbohydrate Metabolism were more abundant for maize starch than for potato (the second most abundance)

and wheat starch (the lowest abundance) (Table S2). Resistant starch is believed to play a key role in SCFA production in the intestine (60). It shifts the SCFA-producing bacteria, resulting in different production of short-chain fatty acids. The relative abundances of *Coriobacteria*, *Holdemanella*, *Acidaminococcus*, *Lactobacillus*, and *Coproccoccus*3, which are SCFA-producing bacteria (61-63), were significantly higher for maize starch. The SCFA production data corroborated the functional prediction results of Carbohydrate Metabolism. The cumulative amount of acetic ($P = 0.006$), propionic ($P = 0.008$), and butyric acid ($P = 0.034$) produced upon maize starch feeding were all significantly higher than for potato starch and wheat starch (Figure 6). These findings suggest that maize starch was more fermentable than potato and wheat starch. These findings, together with the considerable higher abundant carbohydrates metabolism linked to maize starch, suggest a better overall digestive capacity (digestion in the upper GI tract plus fermentation in the colon) of animals fed with maize basal diets. Therefore, a maize basal diet may improve energy absorption in the large intestine in growing pigs. However, further *in vivo* intervention studies need to be performed to validate these hypotheses, for starters with respect to the kinetics of fermentation and SCFA production of the different starches.

In order to investigate which functional composition driven by the different starch sources was more similar to the original pig microbiota, a pig fecal sample was included. Both the PCA and the heatmap of the functional predictive profile showed that the original pig fecal sample clustered with maize starch treatment, which indicated that the functional profile of these samples was more similar to the original pig inoculum (Figure 5).

As discussed above, microbiota fed with maize starch may be more stable or resilient, compared to those of potato and wheat starch, which can more closely simulate the microbial ecology *in vivo*. And the predictive functional metagenome of the microbiota fed with maize starch was more similar to the original inoculum. Thus, maize starch was used as starch source in the recipe of standard ileal effluent medium for pigs (SIEMP). But the microbial composition between microbiota fed with maize starch and the original inoculum still was very different. Figure 1 shows that the original inoculum separated from the microbiota fed with one of the three starch sources, which indicated the different microbial composition between the original inoculum and starch-fed microbiotas. This is reasonable, as pig diets have different carbohydrate sources than human beings. The dominant phyla in the maize starch-fed microbiota were Firmicutes and Actinobacteria, while they were Firmicutes and Bacteroidetes in the original inoculum (Figure 2). Previous reports (1, 7, 8) have also shown that the most abundant phyla were Firmicutes and Bacteroidetes in pig gut microbiota. Therefore, the study II was performed, in which an attempt was made to adjust the microbial composition in the *in vitro* model to become more similar to the original inoculum.

3.2 The Effect of Non-Starch Polysaccharides on Pig Microbiota

Supplementation of non-starch polysaccharides to the SIEMP on top of maize-starch led to increases of the relative abundance of the phyla Bacteroidetes and Proteobacteria, and the decrease of the relative abundance of phylum Actinobacteria (Figure 7A) eventually leading to the competitive exclusion of Actinobacteria. This is supported by the results reported by (12) showing that AX-fed pigs had a higher abundance of species from the *Prevotella* cluster from the phylum Bacteroidetes. (6) found that *Bacteroides* (phylum Bacteroidetes) played a major role in the degradation of AX in children's fecal microbiotas. (64) reported that AX induced a specific increase in *Bacteroides-Prevotella spp.* and *Roseburia spp.* At a lower phylogenetic level, similar results were also found in our study. At the family level, the relative abundances of Prevotellaceae in the microbiota fed with AX and AX-XG were more

than twice times higher compared with that in the microbiota fed with maize starch (Figure 7B). All the three studies mentioned showed that AX can induce increases in *Prevotella* from the family Prevotellaceae (6, 12, 64). There are numerous other studies showing that *Prevotella* is the major genus involved in AX degradation (3, 65-69). AX supplementation also induced the abundance of the family Muribaculaceae from the Bacteroidetes phylum (Figure 7B), which lead to an abundance similar to that of original inoculum. Until now, there is no research about utilization of AX through Muribaculaceae in pigs, but it is known to be a dominant bacterial group in the mouse gut (70). By using 30 population genomes extracted from fecal samples of four different animal hosts (human, mouse, koala, and guinea pig), (71) found that the most abundant glycosidase hydrolases in Muribaculaceae are dominated by xylosidase and arabinosidase enzymes capable of degrading xylan and arabinan, respectively. Thus, we suspect that Muribaculaceae has the capability for degrading AX in the pig gut.

Compared to only supplementing AX to maize starch based SIEMP, microbiota fed AX-XG on top of maize-starch had a higher relative abundance of Bacteroidetes and Firmicutes, and a lower relative abundance of Actinobacteria, which made the microbial composition more similar to the original inoculum (Figure 8A). This indicated that the XG supplementation can further increase a relative abundance of bacteria in the phyla Bacteroidetes and Firmicutes, at the expense of the phylum Actinobacteria. (72) confirmed Bacteroidetes can catabolize XG by a discrete genetic locus, which corroborates that XG supplementation induced the growth of Bacteroidetes in our study. (9) reported that a substantial increase in the *B. uniformis* population occurred after XG addition to an *in vitro* fecal fermentation. At a lower phylogenetic level in our study, XG mainly increased the amount of Prevotellaceae, and led to a slight decrease in the number of Atopobiaceae, Lachnospiraceae, and Muribaculaceae. We did not find any researches about Prevotellaceae catabolizing XG in pigs. But (73) reported that Prevotellaceae from the rumen microbiota can express xyloglucanases, which are XG-degrading enzymes.

Previous results showed that XG generates big microbial community shifts during *in vitro* fecal fermentation (9). Similar results were found in our study; LEfSe results showed that five genera (*Prevotella*, *Lactococcus*, *Succiniclasicum*, *Enterobacter*, and *Escherichia Shigella*) were found more abundantly upon AX-XG feeding compared to AX alone, while only one genus (*Bacillus*) was less abundant compared to AX (Figure S9). Moreover, Figure 8 also shows that microbiota shifts occurred during XG fermentation, compared with AX fermentation. It has been hypothesized that isolated XG, an uncommon carbohydrate structure for microbiota fermentation, has the potential to generate larger shifts in microbiota composition than commonly consumed fibers [AX, mainly in wheat bran] (9). Wheat bran, a by-product from milling industry, contains a large proportion of AX and is indeed widely used in pig industry. However, XG, although existing in almost all land plants, is usually strongly embed in the plant's cellulosic cell wall matrix (74) that may cause it to be difficult to be accessed for bacterial fermentation. In our study, pure (isolated) XG was supplemented to the SIEMP and this was fully accessible to microbiota. This uncommon free form of XG might therefore drive to a different fermentation profile than that of AX-only fed pig microbiota. Interestingly, the facultative anaerobic genus *Bacillus* was found in microbiota fed with AX (average relative abundance, 0.13%). (75) reported that *Bacillus subtilis* was found at all intestinal sites and also in mouse feces, which is also supported by other studies (76, 77). These results demonstrated that *Bacillus* can indeed be part in the gut microbiota.

Supplementation with both AX and XG in maize-based SIEMP also showed higher

reproducibility than only supplementing AX to maize-based SIEMP. Figure S7 shows that microbiota compositions between replicates at different time points upon AX-XG feeding was much more similar than those on AX. In order to test the reproducibility of microbial composition, C.V. of replicates of microbiota fed with AX-XG of alpha diversity, microbial abundance, and microbial activities were evaluated (Table S4, S5, and S6), which were all lower than 15% (expect for 5 taxa in microbial abundance). The low C.V.s indicated that the results of the biological replicates were very reproducible (78). The high reproducibility of TIM-2 (the human version) was previously evaluated by the clustering of the microbiota after the adaptation period (at t0) (79), and during an entire experiment (43). Since SLIM (or TIM-2) is computer-controlled, it is highly reproducible. This has been shown in numerous studies (5, 25, 26, 43).

Overall, at the phylum, family, and genus levels, the microbial composition of AX-XG-fed microbiota was more similar to that of the original inoculum than those of AX-fed and maize starch-fed microbiota (Figure 7, Figure 8A), and the dominant phyla were Firmicutes and Bacteroidetes, which is in agreement with previous reports (1, 7, 8). Even though the microbial composition of the microbiota fed with AX-XG or AX was not exactly the same as the original inoculum, their predictive functional profiles were similar to each other, and clustered together (Figure 8B). Other studies have shown that function structure across microbial community can be remarkably similar, although the taxonomic composition was highly variable (2, 80). This can be explained because many of the same functions can be contained within different species that have independently evolved (2). We also found that microbial composition of samples in AX-XG had less individual differences compared with that in AX (Figure 7). And the swine *in vitro* large intestine model can be reproduced to contain similar microbial communities (table S4, S5, and S6). All the data collectively together, we therefore conclude that both AX and XG were recommended to be supplemented to maize starch based SIEMP, and that the pig gut microbiota fed with this optimized SIEMP had a similar microbial composition and functional profile as the original inoculum.

3.3 Conclusion

In conclusion, different sources of starch significantly changed the pig microbiota composition, ecological network, and predictive functional profile *in vitro*, while two non-starch polysaccharides did not affect this further. In study I, the predicted functional profile of the maize starch group was more similar to the original pig fecal sample, whereas in addition in study II, both the microbial composition and the predictive functional profile of AX-XG groups were more similar to the original swine fecal samples, compared with maize starch and AX alone. Study II showed that the pig microbiota fed with SIEMP (comprising of maize starch, AX, XG, and the rest of the compounds in normal SIEMP) were highly reproducible. All data collectively considered, maize starch with arabinoxylans (AX) and xyloglucan (XG) should be added to the SIEMP recipe to simulate ileal effluent medium for pigs (SIEMP), and this research lays the foundation for experiments mimicking the swine large intestine fermentation, and the possibility to establish the effect of feed interventions on this activity, e.g. with respect to whole intestinal energy extraction of feedstuffs.

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Supplementary Figures:

Figure S1. SLIM schematic, modified from Minekus et al. (1999). a) peristaltic compartments with a hollow fiber membrane inside, b) pH sensor, c) NaOH secretion, d) dialysate system, e) level sensor, f) gaseous N₂ inlet, g) sampling port, h) gas outlet, i) test compound + SIEM feeding syringe, j) temperature sensor.

Supplementary Figure S2. The effect of starch source and fermentation time on alpha diversity indices (Shannon, inverse Simpson and Faith_PD).

Figure S3: Relative abundances of microbial phyla (A) and families (B) in the original

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among maize, potato, and wheat starch treated microbiotas.

Supplementary Figure S5. Co-occurrence networks in the porcine gut microbiome. A. Maize-starch-based network. B. Potato-starch-based network. C. Wheat-starch-based network. Modules were detected by using MCODE in Cytoscape. Each color represents a module. Green edges represent positive interaction, while red ones represent negative interaction, and black ones are unknown.

Supplementary Figure S6. The effect of non-starch polysaccharides and fermentation time on alpha diversity indices (Shannon, inverse Simpson and Faith_PD).

Figure S7. Principal coordinate analysis (PCoA) plots generated based on the calculated distances in a weighted (A) and unweighted (B) UniFrac matrix. Samples were grouped by color (time-point) and shape (in terms of diet group) they belonged to. The ellipses were drawn at the 0.95 confidence level. Weighted: AX to AX-XG ($P = 0.003$), AX-XG to maize starch ($P = 0.003$), AX to maize starch ($P = 0.003$); Unweighted: AX to AX-XG ($P = 0.034$), AX-XG to maize starch ($P = 0.024$), AX to maize starch ($P = 0.141$).

Figure S8: Relative abundances of microbial phyla and families in the original inoculum and microbiota fed with maize starch, AX, and AX-XG.

Supplementary Figure S9. LefSe results on the microbiome of AX and AX-XG group. (A) Histogram of the LDA scores computed for features differentially abundant between AX and AX-XG group. (B) Taxonomic representation of statistically and biologically consistent differences between AX and AX-XG groups. Differences in the most abundance taxa are indicated, red indicating significantly higher in the AX group, green indicating significantly higher in the AX-XG group.

Chapter 3

Pretreatment of rapeseed meal increases its recalcitrant fibre fermentation and alters the microbial community in an *in vitro* model of swine large intestine

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Abstract

The aim of current study was to investigate whether degradation of rapeseed meal (RSM) which was modified by a cellulase, two pectinase, or alkaline treatment was improved by the swine gut microbiota compared to untreated RSM, and whether the microbiota composition was changed. An *in vitro* study was performed to assess how enzymatic and chemical pretreated rapeseed meal (RSM) influences the fibre fermentation and microbial community in the swine large intestine. RSM was processed enzymatically by a cellulase (CELL), two pectinases (PECT), or chemically by an alkaline (ALK) treatment. 16S rRNA gene sequencing data was performed to evaluate changes in the gut microbiota composition, whereas short chain fatty acid production (ion-chromatography) and non-starch polysaccharides (NSP) composition (using monoclonal antibodies; mAbs) were used to assess fibre degradation. The results showed that ALK, CELL, PECT1, and PECT2 changed microbial community composition, increased the abundance of microbial fibre-degrading enzymes and pathways, and increased acetic acid, propionic acid, butyric acid, and total SCFA production. The increased genera also positively correlated with SCFA production. The cell wall polysaccharide structures of RSM shifted after ALK, CELL, PECT1, and PECT2 treatment. The degradation of NSP during the fermentation period was dynamic, and not continuous based on the epitope recognition by mAbs. This study provides the first detailed analysis of changes in the swine intestinal microbiota due to RSM modified by ALK, CELL, PECT1 and PECT2. ALK, CELL, PECT1 and PECT2 altered microbial community structure, shifted the predicted functional metagenomic profile and subsequently increased total SCFA production. Our findings that ALK, CELL, PECT1 and PECT2 increased fiber degradability in RSM could help guide feed additive strategies to improve efficiency and productivity in swine industry. The current study gave insight into how feed enzyme modulate microbial status, which provides good opportunity to develop novel carbohydrase, particularly in swine feed.

Introduction

Rapeseed meal (RSM), a by-product of rapeseed oil production, is not only a suitable protein source for swine feed but also a potentially energy source. RSM contains 20 to 40 % non-starch polysaccharides (NSP) (1, 2). The primary cell walls of RSM consist of pectin and xyloglucan and its cellulose microfibrils are interlinked with xyloglucan via hydrogen bonds forming a stiff network (3). Pectins are linked to each other and cross-linked between pectin and hemicellulose, and between pectin and cellulose (4). Pectins, consisting of homogalacturonan, rhamnogalacturonan, xylogalacturonan, arabinogalactan and arabinan, are the major polysaccharides present in the dehulled rapeseed meal (5). In the secondary cell wall of RSM, the main carbohydrates are 4-O-methylglucuronoxylan, xyloglucan, and cellulose. A drawback of using RSM in animal feed is that the complex cell wall polysaccharides cannot be utilized by endogenous enzymes from monogastric animals (e.g. pigs), and also can only partly be fermented by the microbial community in the gastrointestinal tract (GIT) of the pig.

Therefore, the animal feed sector seeks opportunities to enhance degradability of NSP of feedstuffs, in order to improve its potential as a nutrient source for domestic animals. Previous research showed that physical processing technologies, such as hammer milling, pelleting, wet-milling, extrusion, and mild hydrothermal acid treatment, had limited effect on recalcitrant NSP structures (6, 7). As a result, more efficient solutions are needed to modify the cell wall architecture and allow the gut microbiota to utilize the complex carbohydrates. Former research has shown that NSP-degrading enzymes, such as cellulase and pectinases, could open the cell wall structure and improve NSP degradability (8, 9). Previous studies showed that addition of pectolytic enzymes improved degradability of NSP of RSM *in vitro* (10) and in broilers (11, 12). However, none of the above studies investigated how the gut microbiota was affected by the modified RSM or by the feed enzymes. It is important to know this, as NSP can only be fermented by microbes. Bindelle et al. (13) demonstrated that NSP-degrading enzymes increased abundances of cellulolytic *Ruminococcus*- and xylanolytic *Clostridium*-like bacteria and altered fermentation patterns of barley cultivars and wheat products. Torok and colleagues (14) investigated changes in gut microbial population in response to the supplementation of an NSP-degrading enzyme (containing β -glucanase, xylanase, and protease activities) in a barley-based diet in chickens, and the results showed that microbial composition revealed distinct clusters correlating with un-supplemented and enzyme supplemented birds. Previous research reported that the pretreatment of feed stuffs with carbohydrases can cause the release of reducing sugars and other hydrolysis materials, promoting chemotactic response in specific bacteria, and stimulating their attachment to feed particles, and thereby growth of these microbes (9, 15-17).

In the present study, RSM (predigested with digestive enzymes) was treated independently with two kinds of pectinases (PECT1 and PECT2), one cellulase (CELL), or alkaline (ALK), and afterwards the untreated and treated RSM preparations were fermented in the Swine Large Intestine *in vitro* Model (SLIM) (18). The aim of the current study was to investigate whether fermentation by the swine gut microbiota of treated RSM was improved compared to untreated RSM, and whether the microbiota composition and activity were changed.

Materials and methods

Substrate preparation

Rapeseed meal (*Brassica napus*, Cargill N.V., Antwerp, Belgium; 2011) was obtained from a commercial feed mill (Agrifirm B.V., Utrecht, the Netherlands). Preparation method I (predigestion of RSM after carbohydrase or alkaline treatment) [Figure 1]: to 200 g of RSM 40 mL 10*gastric electrolyte concentrate solution (GES, 310 g sodium chloride, 110 g potassium chloride, 15 g calcium chloride di-hydrate, and 4840 g ultrapure water) and 360 mL ultrapure water were added. The pH was adjusted to 5.5 and then nothing (CON), 10 mL of alkaline (ALK, 6 M NaOH), or the following carbohydrases were added CELL (Accellerase 1000, Sigma-Aldrich, Missouri, United States), PECT1 (Pectinex Ultra SP, Novozymes A/S, Bagsvaerd, Denmark), or PECT2 (Multifect Pectinase, DuPont Industrial Biosciences, Genencor division, Rochester, NY). Enzyme preparations were incubated at 37 °C for 2 h, with occasional shaking (every 30 mins), while ALK was incubated overnight at 4 °C. Enzyme preparations were then heated at 100 °C for 5 min to inactive enzymes. Afterwards, for all five samples, 120 mL GES was added and pH adjusted to 3 to continue with the gastric incubation according to the predigestion protocol as described elsewhere (19). After predigestion, the slurry was centrifuged (8.000 g, at 4 °C, for 20 minutes) and dialysis was performed for the supernatants. For dialysis, a dialyzer (Sureflux, Nipro Europe Group Companies, Mechelen, Belgium) was used with a peristaltic pump to remove small digestion products and water. After reduction of the total volume to ~450-500 mL, supernatant was mixed with pellet, and freeze-dried. Method II (predigestion of RSM before carbohydrase or alkaline treatment) [Figure 1]: four batches of 200 g RSM were predigested as described before (19) and then dialyzed. Afterwards, 55 mL 10*GES was added, and pH adjusted to 5.5, after which 10 mL of CELL, PECT1, PECT2, or ALK treatment commenced, respectively. Enzyme preparations were incubated at 37 °C for 2 hours with occasional shaking (every 30 mins), and ALK was incubated overnight at 4 °C. Afterwards, enzyme preparations were heated at 100 °C for 5 min to inactive enzymes, and pH was neutralized to 6.5-7 with HCl or NaOH, and the samples were freeze-dried. Samples are differentiated by the suffix _B (for before) or _A (for after) (e.g. PECT1_A) for carbohydrase- or ALK-treatment prior to and after digestion, respectively.

Figure 1. Schematic experimental setup for fibre fermentation in the Swine Large Intestine

in vitro model (SLIM).

Fermentation in The Swine *in vitro* Large Intestinal Model (SLIM)

The setup of SLIM was as follows: a fully computer-controlled *in vitro* model based on TIM-2 (20) was used to mimic the swine large intestine (18). The pH (5.9) was controlled by continuous addition of 2 M sodium hydroxide. Standard ileal efflux medium of pigs (SIEMP) was used to simulate the materials entering the colon. The SIEMP, adapted from (21) and described in Long et al.(2020) (18) contained the following components (g/L): 74.6 maize starch, 9.0 xylan, 19.0 pectin, 9.0 amylopectin, 9.0 arabinogalactan, 9.0 arabinoxylan, 9.0 xyloglucan, 31.5 Tween 80, 43.7 casein, 0.7 ox-bile, 43.7 bactopecton, 4.7 K₂HPO₄·3H₂O, 0.009 FeSO₄·7H₂O, 8.4 NaCl, 0.8 CaCl₂·2H₂O, 0.7 MgSO₄·7H₂O, 0.05 bile, 0.02 haemin and 0.3 cysteine·HCl, plus 1.5 mL of a vitamin mixture containing (per litre): 1 mg menadione, 0.5 mg vitamin B12, 2 mg D-biotin, 10 mg pantothenate, 5 mg p-aminobenzoic, 4 mg thiamine and 5 mg nicotinamide acid. The pH was adjusted to 5.9. Dialysis liquid contained (per litre): 2.5 g K₂HPO₄·3H₂O, 0.005 g FeSO₄·7H₂O, 4.5 g NaCl, 0.45 g CaCl₂·2H₂O, 0.05 g bile, 0.5 g MgSO₄·7H₂O and 0.4 g cysteine·HCl, plus 1 mL of the vitamin mixture. All medium components were purchased at Tritium Microbiology (Eindhoven, The Netherlands). The pig fecal inoculum was a standardized microbiota from growing pigs, freshly collected from the floor (48 pens with 6 pigs/pen, Hypor Libra x Hypor Maxter, Hendrix Genetics, Boxmeer, The Netherlands), but only material from the top was selected (so not toughing the floor). Feces was pooled and mixed with dialysate as described before (18).

In order to create a complete anaerobic environment, SLIM with 90 mL dialysate in each of the 4 individual units was flushed with gaseous nitrogen for at least 3 hours before incorporating the standardized microbiota. Thirty mL of the standardized microbiota was added to each SLIM-unit, making the total volume 120 mL. Figure 1 shows the experimental set-up for fibre addition to SLIM. The microbiota was adapted to the model with SIEMP for 16 hours. During the adaptation phase, SIEMP was added into each SLIM-unit at a rate of 2.5 mL/h through the feeding syringe. At the end of the adaptation period, a 2-hour starvation period was performed, which was used to allow all the carbohydrates within SIEMP to be fermented. Afterwards, a shot of 5 grams of the different RSMs was given to the system at time point 0 hours, and incubation was continued for 24 h after that (Figure 1).

Sample collection

Samples from lumen and spent dialysate were collected at time point 0, 0.5, 1, 2, 4, 6, 8, and 24 h (t0, t0.5, t1, t2, t4, t6, t8, and t24). They were snap-frozen in liquid nitrogen and stored until analyses. Lumen samples were used to analyze microbiota composition and polysaccharides structures, and both lumen and dialysis samples were analyzed for short chain fatty acid (SCFA) concentrations.

Sequencing of the V3-V4 region of the 16S rRNA gene

Microbial DNA extraction and sequencing of the V3-V4 region of the 16S rRNA gene were performed by BaseClear B.V. (Leiden, The Netherlands). Briefly, genomic DNA extraction was performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, California, USA) according to the manufacturer's instructions. Barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step PCR. 10-25 ng genomic

DNA was used as template for the first PCR with a total volume of 50 μ L using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers (22) appended with Illumina adaptor sequences. PCR products were purified (QIAquick PCR Purification Kit, Venlo, The Netherlands) and the size of the PCR products were checked on a Fragment analyzer (Advanced Analytical, Ankeny, US) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina, city, CA, USA). Subsequently, PCR products were purified, checked on a Fragment analyzer and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) and demultiplexed based on sample-specific barcodes.

Bioinformatics analysis

The demultiplexed raw sequences obtained from BaseClear were processed using the QIIME2 pipeline (23). In short, reads were imported and quality filtered and dereplicated with q2-dada2 (24). Next, dada2 was performed with paired-end reads and truncations parameters were as follows: the first 17 and 14 base pairs were trimmed off in forward and reverse reads, respectively. And at position 280 base pairs the fragment was truncated in forward reads, and at position 230 base pairs for the reverse reads. The processed sequences were used for all the downstream analyses. Alpha-diversity (Shannon index) and β -diversity (weighted and unweighted UniFrac) were analyzed by the q2-phylogeny plugin (<https://github.com/qiime2/q2-diversity>).

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, PICRUST2. The PICRUST2 software (25) was used to predict microbial functional abundances based on marker gene sequences. KEGG database was used to predict the results.

Chemical analyses

Short-chain fatty acids analyses. Samples from lumen and dialysate were analyzed by Brightlabs (Venlo, The Netherlands) for determination of concentrations of SCFA. Ion exclusion chromatography (IEC) was applied on an 883 Ion Chromatograph (IC; Metrohm, Switzerland), using a Transgenomic IC Sep ICE-ION-300 column (30 cm length, 7.8 mm diameter and 7 μ m particles) and a MetroSep RP2 Guard. The mobile phase consists of 1.5 mM aqueous sulphuric acid. Samples were centrifuged (21,000 g, 10 min) and the clear supernatant was filtered through a 0.45 μ m PTFE filter and diluted with mobile phase (for lumen 1:5, for dialysate 1:2). Ten microliters were loaded on the column by an autosampler 730 (Metrohm). Molecules were eluted according to their pKa. A column flow rate of 0.4 ml*min⁻¹ was used. The temperature of the column was 65 °C. The organic acids were detected using suppressed conductivity detection

Glycome profiling

Sample preparation. Lumen samples from each time point and treatment were freeze-dried, after which they were dissolved at 1 mg/mL in deionized water, and stored at -20 °C as stock solutions.

Monoclonal antibodies (mAbs). mAbs were obtained as hybridoma cell culture supernatants from CarboSource (Atlanta, GA, USA) [<http://www.carbosource.net/>].

ELISA. The ELISA protocol was slightly modified from Pattathil et al. (2010) (26). In brief, samples prepared above were applied (50 μ L of 100 μ g/mL in deionized water per well) to Costar 3598 96-well plates (Corning Life Sciences, city, country) and were dried to the well surfaces by evaporation overnight at 37°C. Control wells contained deionized water. The plates were blocked with 200 μ L of 1% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (50 mM Tris-HCl, pH 7.6, containing 100 mM sodium chloride) for 1 h. Blocking agent was removed by aspiration, and 50 μ L of undiluted hybridoma supernatant were added to each well and incubated for 1 h at room temperature. Supernatant was removed and wells were washed three times with 200 μ L of 0.1% (w/v) BSA in Tris-buffered saline (wash buffer). Peroxidase-conjugated goat anti-mouse IgG, anti-mouse IgM, goat anti-rat IgG, or goat anti-rat IgM antibodies (Sigma-Aldrich), depending on the primary antibody used, were diluted 1:5,000 in wash buffer, and 50 μ L were added to each well and incubated for 1 h. Wells were then washed five times with 200 μ L of wash buffer. Next, 3,3',5,5'-tetramethylbenzidine (TMB) solution (Sigma-Aldrich, St. Louis, USA) was freshly prepared according to the manufacturer's instructions, and 50 μ L were added to each well. After 20 min, the reaction was stopped by adding 50 μ L of 0.5 N sulfuric acid to each well. The OD of each well was read at a wavelength of 450 nm using a Multi-mode microplate reader (BioTek Synergy HTX, Abcoude, The Netherlands).

Polysaccharide panel screening. Polysaccharide panel screening of mAbs was carried out by ELISA against all lumen samples immobilized to 96-well plates. Duplicate preparations of each polysaccharide were used for all experiments reported here. Binding data was visualized in R via ComplexHeatmap package (27).

Statistics

Kruskal-Wallis Rank Sum Test (one-way ANOVA on ranks) was applied to compare α -diversities (Shannon index) among different RSM treatments and time points, and Wilcoxon Rank Sum Test was used for pairwise comparison in R version 3.5.3 (<https://www.r-project.org/>). Bonferroni was used to correct *P*-values. Permutational multivariate analysis of variance (PERMANOVA; REFERENCE) was performed to test the significance of β -diversity distances in QIIME2 (weighted and unweighted UniFrac) between non-processed and processed RSM. The results were visualized in R (R version 3.5.3).

The amplicon sequence variant (ASV) table (feature table of QIIME2) was normalized and filtered in R, and statistical analysis and visualizations were performed in R and STAMP (Parks, Tyson et al. 2014). The table was normalized via division by the sum of sequences in a given sample and multiplied by the minimum sum across all samples. Relative abundances were filtered as follows: values below a relative abundance threshold of 0.01% were not taken into account; taxa with a median relative abundance <1 % in all groups were not considered for statistical analysis. White's non-parametric t-test was applied to compare between CON and treatments. *P*-values were corrected using the Benjamini-Hochberg method. A *q*-value (corrected *P*-value) < 0.05 was considered significant.

Pearson correlations between continuous meta-variables and taxonomic variables were calculated and visualized in R. Parameters were set as follows: Missing values for meta-variables were handled as NO imputation (replacing missing data with substituted); zeros were kept for the calculation of correlation; a minimum number of 0.1 % was considered for calculation; a minimum of 4 paired observations were required for calculation of correlations.

P-values were corrected using the Benjamini-Hochberg method. A *q*-value (corrected *P*-value) < 0.05 was considered significant.

SCFA production between CON and the treated RSM substrates were compared and visualized in R.

Results

Characteristics of non-processed and processed RSM

A comprehensive set of 155 plant cell wall glycan-directed monoclonal antibodies was used to screen untreated RSM by a ELISA-based assay (26, 28), and 34 antibodies reacted with RSM (data not shown). These were subsequently used in the current study to obtain information on the presence and relative abundance of specific epitopes that are characteristic of the different types of polymers in untreated RSM and RSM processed by ALK, CELL, PECT1, and PECT2.

Figure 2 shows that both increases and decreases in epitope recognition occurred in ALK, CELL, PECT1, and PECT2 compared to CON. Samples from after (_A) and before (_B) predigestion clustered together according to each treatment, which indicated _A and _B from the same treatment had similar epitope accessibility. ALK strongly increased binding of non-fucosylated XG mAbs, while CELL, PECT1, and PECT2 led to disappearance of those compared to CON, regardless of _A and _B treatment. All the treatments increased the binding of “Linseed Mucilage RG-I group” directed mAbs, but had little effect on Xylan-2 and RG-Ic group compared to CON. Binding of MAC204 (AG-1), which is binding to gum tragacanth and to lettuce and green tomato RG-I preparations (arabinogalactan), disappeared with ALK_A, ALK_B, and CELL_B, while increased binding of CCRC-M107 (AG-2), which binds to linear and branched arabinans and RG-I preparations from diverse plants but does not bind to larch arabinogalactan (26), was observed in ALK_A and ALK_B. CELL_A, CELL_B, PECT1_A, PECT1_B, and PECT2_B led to disappearance of the binding of mAbs of “pectic backbone group” and CCRC-M 133, which also binds to linear and branched arabinans and RG-I preparations from diverse plants but do not bind to larch arabinogalactan. ALK, PECT1, and PECT2 increased binding of mAbs directed against the arabinogalactan side chains of RG-I (RG-I/AG).

Figure 2. Glycome profiling of non-processed and processed RSM. The binding response data of the mAbs are presented as heatmap using a blue-white-red scale indicating the intensity of the ELISA signal (blue, white, and red colors depict no, medium, and strong binding, respectively). The mAbs (indicated by their codes) are grouped based on the cell wall glycans they predominantly recognize as shown in the panel on right-hand side of the figure. _A, RSM was treated after predigestion, _B; RSM was treated before predigestion. XG, xyloglucan; RG-I, rhamnogalacturonan I; AG, arabinogalactan; RG-I/AG, arabinogalactan side chains of RG-I.

ALK, CELL, PECT1, and PECT2 significantly changed microbiota composition compared to CON

To determine the changes in composition of the gut microbiota fed with a shot of 5 g CON, ALK, CELL, PECT1, or PECT2, a comparison of microbiota based on sequencing the V3-V4 region of the 16S rRNA gene was performed. Shannon indices significantly decreased at t4, t6, t8 and t24, compared to that of t0 (Figure S1A). When data from all of the time points were pooled, there were no significant differences among CON, ALK, CELL, PECT1, and

PECT2 in Shannon index (Figure S1B). Phylogeny based UniFrac methodology was then used to compare the β -diversity of the microbial communities between microbiota fed with non-processed and processed RSM. Unweighted UniFrac analysis (Figure 3) shows that samples from processed RSM (ALK, CELL, PECT1, and PECT2) significantly ($P = 0.004$) separated from non-processed RSM (CON), and samples from different processed method clustered together. Samples from CON, ALK, CELL, PECT1, and PECT2 all clustered together ($P = 0.125$) with respect to weighted UniFrac (Figure S2).

Figure 3. Principal coordinate analysis (PCoA) plot generated based on the calculated distances in unweighted UniFrac matrix. Samples were grouped by shape and color in terms of treatment and time point they belonged to, respectively: CON (sphere), ALK (plus), PECT1 (triangle), PECT2 (diamond), CELL (square); a red-green-purple scale was used to indicate the fermentation time (red and purple depict start and end of the fermentation period).

There were no significant differences between microbiota fed with RSM predigested before

or after carbohydrase or ALK treatment with respect to both α -diversity (Figure S3), or β -diversity (data not shown), which indicated predigesting before or after processing RSM had little effect on microbiota composition. Glycome profiling of RSM (Figure 2) also shows that samples from after and before processing clustered together according to each treatment, which indicated their polysaccharide compositions were similar to each other. Therefore, they were treated as duplicates in the following (microbial relative abundance) analyses.

Relative abundances of taxa within the pig microbiotas fed with non-processed and processed RSM were compared to identify significantly different bacterial taxa. At genus level (Figure 4), seven genera were significant higher in relative abundance after ALK, CELL, PECT1, and PECT2 treatment compared to CON. These were *Ruminococcaceae NK4A214* group, *Ruminococcaceae UCG-002*, *Ruminococcaceae UCG-005*, *Roseburia*, *Anaerotruncus*, *Bifidobacterium*, *Christensenellaceae R-7* group, and *Selenomonas*. For genera *Christensenellaceae R-7* group and *Ruminococcaceae UCG-005*, their relative abundances were also higher in ALK and PECT1 compared to CELL. Instead, the relative abundances of *Prevotella 7*, an unclassified genus from *Prevotellaceae*, and *Prevotellaceae UCG-001* were significantly decreased after feeding ALK, CELL, PECT1, and PECT2 compared to microbiota fed with CON. The relative abundance of *Succinivibrionaceae UCG-001* was significant higher in ALK and PECT1 compared to CON.

Figure 4. Significantly different relative abundances of microbial genera upon treatment with ALK, CELL, CON, PECT1, and PECT2. * $P < 0.05$; ** $P < 0.01$

PICRUSt2 analyses revealed that microbial functional abundances related to carbohydrate metabolism and SCFA production were significantly increased with processed RSM compared to CON

PICRUSt2 was performed to the 16S rRNA gene data to predict metagenomic functional profiles. In this study we focused on carbohydrate metabolism related microbial functions

(Figure 5). The relative abundances of fibre degradation pathways, beta-glucosidase [EC:3.2.1.21], beta-mannosidase [EC:3.2.1.25], cellobiose phosphorylase [EC:2.4.1.20], and sucrose phosphorylase [EC:2.4.1.7], were significant higher in ALK, CELL, PECT1, and PECT2 compared to CON, whereas conversely that of alpha-L-fucosidase [EC:3.2.1.51] was significant higher in CON compared to ALK, CELL, PECT1, and PECT2. For cellobiose phosphorylase [EC:2.4.1.20] and sucrose phosphorylase [EC:2.4.1.7], the relative abundances in ALK were also significant higher than those of CELL, PECT1, and PECT2.

Figure 5. Significantly different metagenomic functions in relative abundance among ALK, CELL, CON, PECT1, and PECT2. * $P < 0.05$; ** $P < 0.01$.

Six microbial pathways related to fermentation were significant higher in relative abundance when microbiotas were fed with ALK, CELL, PECT1, and PECT2 compared to microbiota fed with CON. These pathways were pyruvate dehydrogenase E1 component, short-chain fatty acids transporter, mannose-1-phosphate guanylyltransferase, superpathway of glucose and xylose degradation, sucrose degradation IV, and L-lysine fermentation to acetate and butanoate. The relative abundance of lactose/L-arabinose transport system permease protein was significant higher in ALK, PECT1 and PECT2 compared to CON.

Figure 6 shows that the cumulative acetic, propionic, and butyric acid and total SCFA production were higher in ALK, CELL, PECT1, and PECT2 compared to CON. For acetic acid, more than 2 times greater production was observed when the microbiota was fed with ALK, CELL, PECT1, and PECT2 compared to when the microbiota was fed with CON. The production of propionic, butyric acid and total SCFA in ALK, CELL, PECT1, and PECT2 were more than 1.6 times higher than that in CON, except for propionic (1.3 times), and butyric acid (1.4 times) production in ALK.

Treatment	acetic.acid	propionic.acid	butyric.acid	total
ALK	2.0	1.3	1.6	1.4
CELL	2.3	1.7	1.8	1.7
PECT1	2.2	1.6	1.6	1.6
PECT2	2.5	1.9	1.8	1.8

Figure 6. Cumulative Acetic (A), Propionic (B), and Butyric acid (C), and total short-chain fatty acid (D) production during fermentation of ALK, PECT1, PECT2, and CELL compared to CON. Fold change of short-chain fatty acid in ALK, PECT1, PECT2, and CELL compared to CON (E).

Glycome profiling shows that binding of mAbs in lumen digests were dynamic during the *in vitro* fermentation in SLIM

To investigate the dynamic changes of polysaccharides structure in CON, ALK, CELL, PECT1, and PECT2 during *in vitro* fermentation, a time series of sampling was performed and the set of 34 mAbs was used to screen the lumen digests. Figure 7 shows that no or few binding signals were observed in Non-fucosylated XG, AG-2, and Pectic Backbone mAbs upon feeding CON, ALK, CELL, PECT1, and PECT2 at all time points.

Figure 7. Glycome profiling of lumen digests during *in vitro* fermentation of ALK, CELL, CON, PECT1, and PECT2 at time point 0, 0.5, 1, 2, 4, 6, 8, and 24 h. The binding response data are presented as heatmap using a blue-white-red scale indicating the strength of the ELISA signal (blue, white, and red colors depict no, medium, and strong binding, respectively). The mAbs are grouped based on the cell wall glycans they predominantly recognize as shown in the panel on the right-hand side of the figure. XG, xyloglucan; RG-I, rhamnogalacturonan I; AG, arabinogalactan; RG-I/AG, arabinogalactan side chains of RG-I.

No binding of CCRC-M83 that specifically bind to Linseed Mucilage RG-I was observed at t0 in all treatments (just prior to addition of the fibre shots), and binding signals appeared

afterwards. For ALK, binding of CCRC-M83 decreased from t0.5 to t2, increased again at t4, and decreased to the lowest value at t24. For CELL, binding of CCRC-M83 decreased from t0.5 to t2, stabilized from t4 to t8, and decreased to the lowest value at t24. For CON, binding increased from t0.5 to t1, decreased from t2 to t6, increased at t8, and decreased to the lowest value at t24. For PECT1, binding increased from t0.5 to t4, and then decreased progressively until t24. For PECT2, binding increased slightly from t0.5 to t8, and then decreased to the lowest value at t24.

In terms of Xylan-2 recognizing mAb (i.e. CCRC-M105), weak bindings were detected at all time points after t0 with CELL and CON. Increased binding of CCRC-M105 was observed from t0.5 to t1 in ALK and the binding strength decreased from t2 to t4, slightly increased again at t6, and then decreased until t24. For PECT1, increased binding of CCRC-M105 was observed from t0.5 to t6, which decreased afterward until t24. Binding for PECT2 was dynamic from t0.5 to t24, but the lowest binding was observed at t24.

As for the RG-Ic recognizing mAb (i.e. CCRC-M30), binding of CCRC-M30 was lower in ALK, PECT1, and PECT2 according to each time point compared to CELL and CON, but binding over time was dynamic. With respect to the AG-4 mAbs (recognizing arabinogalactans), weak and dynamic binding was observed at each time point in all treatments.

With respect to RG-I/AG mAbs, more active mAbs were observed compared to other groups of mAbs in all treatments. Within RG-I/AG mAbs, binding strengths of CCRC-M25 and CCRC-M60 were stronger than other RG-I/AG mAbs in all treatments, and their binding strengths were fluctuating during the whole fermentation period and still existed at t24 in all treatments.

Correlation between microbiota abundance and SCFA production and mAb binding

Pearson correlation analyses were performed to investigate the relationship between the relative abundance of microbial genera and SCFA production at each time point (Figure 8). Seven genera (*Bifidobacterium*, *Collinsella*, *Denitrobacterium*, *Olsenella*, *Coriobacteriaceae.1*, *Bacteroidales S24-7 group.2*, and *Acetitomaculum*) had significant negative correlation with propionic acid, butyric acid, valeric acid and total SCFA production. Within these, *Bacteroidales S24-7 group.2*, *Olsenella*, *Coriobacteriaceae.1*, and *Acetitomaculum* also significantly negatively correlated with acetic acid. Eight genera (*Bacteroidales S24-7 group.1*, *Prevotella 9*, *Faecalibacterium*, *Ruminococcaceae UCG-005*, *Ruminococcus 2*, *Selenomonas*, *Succinivibrio*, and *Succinivibrionaceae UCG-001*) significantly positively correlated with acetic acid, propionic acid, butyric acid, valeric acid and total SCFA production. Within these, *Ruminococcus 2* and *Succinivibrio* also had significant positive correlation with caproic acid. *Bacteroidales S24-7 group*, *Sarcina*, and *Oribacterium* had significant positive correlation with propionic acid, butyric acid, valeric acid and total SCFA production. *Roseburia*, *Ruminococcaceae NK4A214 group* and *Ruminococcaceae UCG-002* significantly positively correlated with acetic acid, propionic acid, butyric acid and total SCFA production. *Prevotella 7* significantly positively correlated with propionic acid, valeric acid, caproic acid, and total SCFA production.

Figure 8: Correlation between core bacterial genera, and SCFA and binding of mAbs. Statistical significance was determined for all pairwise comparisons using Pearson's method. * $P < 0.05$; ** $P < 0.01$. Size of the circles indicates correlation strength, which bigger sizes meaning higher correlations. Blue circles represent positive correlations (correlation coefficients from 0 to 1), whereas red circles represent negative correlations (correlation coefficients from 0 to -1). total, total SCFA production. The mAbs are grouped based on the cell wall glycans they predominantly recognize as shown in the panel on the left-hand side of the figure. XG, xyloglucan; RG-I, rhamnogalacturonan I; AG, arabinogalactan; RG-I/AG, arabinogalactan side chains of RG-I.

The correlation between binding of mAbs and relative abundance of microbial genera was also analyzed. Within the mAbs that recognizing non-fucosylated XG, CCRC-M93 had significant negative correlation with *Bacteroidales S24-7 group.1*, *Christensenellaceae R-7 group*, [*Eubacterium*] *nodatum group*, *Blautia*, *Lachnospira*, *Lachnospiraceae NK3A20 group*, *Roseburia*, *Ruminococcaceae UCG-005*, *Ruminococcus 2*, *Subdoligranulum*, *Catenibacterium*, *Catenisphaera*, *Succiniclasticum*, and *Succinivibrionaceae UCG-001*. CCRC-M96 significantly negatively correlated with *Bacteroidales S24-7 group.2* and *Acetitomaculum*, while it significantly positively correlated with *Anaerotruncus*. CCRC-M99 had significant negative correlation with *Dorea* and *Ruminococcus 2*, whereas it had significant positive correlation with *Megasphaera*. CCRC-M104 had significant positive correlation with *Prevotellaceae UCG-001*, while it negatively correlated with *Ruminococcaceae UCG-002*.

MH4.2A4 that recognizes linseed mucilage RG-I had significant negative correlations with *Bacteroidales S24-7 group.1*, *Ruminococcaceae NK4A214 group*, and *Ruminococcaceae UCG-005*.

CCRC-M105 that binds to Xylan-2 had significant negative correlations with *Prevotellaceae*.1, *Family XIII UCG-001*, *Acidaminococcus*, and *Megasphaera*, whereas it positively correlated with *Ruminococcus* 2 and *Subdoligranulum*.

CCRC-M30, binding to RG-Ic, had significant positive correlations with *Denitrobacterium*, *Olsenella*, *Bacteroidales S24-7* group.2, and *Acetitomaculum*, while it significantly negatively correlated with *Roseburia*, *Faecalibacterium*, *Ruminococcaceae UCG-002*, *Ruminococcaceae UCG-005*, *Selenomonas*, and *Succinivibrionaceae UCG-001*.

MAC204, that recognizing AG-1, significantly negatively correlated with *Denitrobacterium*, *Olsenella*, *Bacteroidales S24-7* group.2, *Acetitomaculum*, and *Roseburia*, while it had significant positive correlations with *Lactobacillus*, *Sarcina*, *Roseburia*, *Ruminococcaceae NK4A214* group, *Ruminococcaceae UCG-002*, *Ruminococcaceae UCG-005*, and *Selenomonas*.

CCRC-M133, binding to AG-2, significantly negatively correlated with *Denitrobacterium*, *Prevotella* 7, *Prevotellaceae UCG-001*, *Acetitomaculum*, and *Subdoligranulum*, while it positively correlated with *Ruminococcaceae UCG-002*.

CCRC-M131, that binds to pectic backbone, had significant negative correlation with *Denitrobacterium*, *Olsenella*, *Bacteroidales S24-7* group.2, *Prevotellaceae UCG-001*, *Acetitomaculum*, and *Subdoligranulum*, while it positively correlated with *Lactobacillus*, *Anaerotruncus*, and *Ruminococcaceae UCG-002*.

With respect to mAbs binding to AG-4, CCRC-M78 had significant negative correlation with *Bifidobacterium*, and *Christensenellaceae R-7* group, whereas it significantly positively correlated with *Prevotellaceae UCG-001*. CCRC-M91 had significant negative correlation with *Denitrobacterium*, *Olsenella*, and *Acetitomaculum*, and CCRC-M91 and CCRC-M92 significantly positively correlated with *Lactobacillus*. JIM13 had significant negative correlation with *Subdoligranulum*.

As for mAbs binding to RG-I/AG, they had significant negative correlation with *Bacteroidales S24-7* group (CCRC-M32), *Bacteroidales S24-7* group.2 (CCRC-M32 and M125), *Lactobacillus* (CCRC-M24), [*Eubacterium*] *nodatum* group (CCRC-M60), *Acetitomaculum* (CCRC-M42), *Blautia* (CCRC-M60), *Coprococcus* 3 (CCRC-M24), *Ruminococcaceae NK4A214* group (CCRC-M32, M41, M44, and M60), *Ruminococcaceae UCG-002* (CCRC-M44), *Ruminococcaceae UCG-005* (CCRC-M32, M44, and M60) *Subdoligranulum* (CCRC-M 42), and *Succiniclasticum* (CCRC-M125). Significantly positively correlations were observed with *Olsenella* (CCRC-M44), *Bacteroidales S24-7* group (CCRC-M128), *Prevotella* 7 (CCRC-M24 and M128), *Prevotellaceae UCG-001* (CCRC-M 24 and M128), *Prevotellaceae* (CCRC-M128), *Lactobacillus* (CCRC-M42), *Lachnospira* (CCRC-M128), *Ruminococcus* 2 (CCRC-M128), *Subdoligranulum* (CCRC-M128), and *Succinivibrio* (CCRC-M128).

Discussion

Cell wall polysaccharides composition of differently pretreated RSM

Cell wall polysaccharides of RSM (*Brassica napus*) were comprehensively studied by chemical methods (29), which showed that they generally consisted of arabinan,

homogalacturonan, Rhamnogalacturoan I (RG-I), type II arabinogalactan (AG), and xyloglucan (XG) (30). In a previous study (Long, de Vries and Venema submitted), we determined the monosaccharide constituent composition, which was in line with Pustjens et al. (2013). Our current findings with the mAbs are also in line with this, since nonfucosylated XG-, RG-I-, pectic backbone-, and AG-recognizing mAbs bound to CON. By similar reasoning, xylan was also detected in CON, which was not reported before, although it is not entirely clear how much cross-reactivity the mAbs show.

ALK treatment intensively increased binding of mAbs that specifically bind to nonfucosylated XG (Figure 2). This is consistent with previous finding that alkali could extract XG from hemicellulose (29), which led to XG being detected by the mAbs in the current study. Previous research also demonstrated that XG is linked via hydrogen bridges to the surface of cellulose microfibrils, which is extractable with alkali, but not accessible by enzyme (31). However, enzymatic treatments (PECT1, PECT2, and CELL) led to a disappearance of binding nonfucosylated XG-specific mAbs compared to CON, which was unexpected. It could be that XG might become entrapped by other cell wall structures after pectinase or cellulase treatment. It is unknown whether this kind of the hidden phenomenon would reduce the degradability of XG.

Enzymatic treatments increased binding of linseed mucilage RG-I-specific mAbs and some mAbs directed against the arabinogalactan side chains of RG-I (RG-I/AG), and reduced binding of some mAbs directed to pectic backbone, which indicated that PECT1 and PECT2 broke down pectic backbone and exposed RG-I and its arabinogalactan side chains. These results are supported by a previous study showing that PECT1 and PECT2 degraded pectic backbone and released RG-I and its arabinogalactan side chains (29). Moreover, RG-I attached to cellulose microfibrils can be released by cellulase (32), while at the same time it might block the accessibility of pectic backbone because of the shifting of the polysaccharides structure.

ALK treatment led to the disappearance of binding of mAbs that specifically bind to AG-1 while it increased binding of mAbs that specifically binding to AG-2, which indicated that ALK could cause arabinogalactan to be physically entrapped in other cell wall structures, while linear and branched arabinan become accessible (26).

They were lower in binding strengths of mAbs in AG-4 from ALK_B, PECT1_B, PECT2_B, and CELL_B, compared to ALK_A, PECT1_A, PECT2_A, and CELL_A, respectively. This indicated that small fragments were produced in AG-4 group after enzymatic and chemical treating RSM, which were dialyzed out in ALK_B, PECT1_B, PECT2_B, and CELL_B during the subsequent predigestion treatment. Overall, both enzymatic and chemical processed RSM can release some polysaccharides but physically entrap or shield some others at the same time.

Community structure of swine microbiota fed with differently pretreated RSM

The PCoA analysis showed significant difference of both enzymatic and chemical treatments on swine microbial community ($P = 0.004$) compared to CON, based on unweighted UniFrac metric (Figure 3); and overall there were no significant differences in this metric between treatments (Figure 3). Dietary fibre is known to have a considerable effect on gut microbiota composition (33-35). Glycome profiling of non-processed and processed RSM showed that

polysaccharides structures were differentially shifted due to ALK, PECT1, PECT2, and CELL treatment in the current study, which were consistent with previous studies (10, 12). These observations might explain the specific genus changes in microbiota composition after microbiotas fed with ALK, PECT1, PECT2, and CELL compare to CON (Figure 4), despite the differential effect of the different treatments as assessed by the glycome profiling.

In order to understand which microbes were significantly influenced by dietary supplementation of processed RSM, microbial genera were compared among the five treatments (Figure 4). The relative abundances of *Ruminococcaceae* NK4A214 group, *Ruminococcaceae* UCG-002, *Ruminococcaceae* UCG-005, *Roseburia*, *Bifidobacterium*, and *Christensenellaceae* R-7 group were significantly increased in ALK, PECT1, PECT2, and CELL (Figure 4). Research has shown that genera of family *Ruminococcaceae* contain major (hemi)cellulolytic and pectinolytic species (36-38). Thus, microbes from family *Ruminococcaceae* play an important role in degrading (hemi)cellulose and pectin in their activity against recalcitrant fibre (39, 40). Previous research demonstrated that the higher the relative abundance of *Ruminococcaceae* NK4A214 group, the higher the fibre degradability (37, 41). This observation was consistent with the current study, which showed that the SCFA production (which is usually used as evaluation of fibre degradability *in vitro*) was also higher in ALK, CELL, PECT1, and PECT2 compared to CON. Furthermore, our study also showed that the relative abundance of *Ruminococcaceae* NK4A214 group, as well as *Ruminococcaceae* UCG-002 and *Ruminococcaceae* UCG-005, significantly correlated with acetic acid, propionic acid, butyric acid, and (not surprisingly) total SCFA production. *Roseburia* is a well-known butyrate-producing bacteria (42), and a primary degrader of β -mannans (43). The significant increase in abundance of this genus was accordance with the increased butyric acid production in the current study (Figure 6), and increased predicted abundance of beta-mannosidases (EC.3.2.1.25) in the processed RSM groups (Figure 5). *Bifidobacterium* is suggested to be a common cross-feeding bacteria for sugar utilization (44). Previous research has been shown that *Bifidobacterium bifidum* relies on the presence of a primary degrader in order to grow with either resistant starch or xylan both *in vitro* (45) and *in vivo* (46). It has also been reported that numerous *Bifidobacterium* species grew to higher cell densities accompanied by upregulating their respective saccharolytic pathways when grown in co-culture compared to their growth in monoculture (47). This finding was consistent with the current study, where the relative abundance of *Bifidobacterium* was much higher than that of other genera, especially in ALK (data not shown). Alternatively, synergy existed among genera that were targeting the same substrates, possibly by specializing in degrading different motifs within the molecule.

The relative abundance of *Christensenellaceae* R-7 group increased after enzymatic and chemical treatment, which was consistent with a previous study where rumen microbiota was fed with fibrolytic enzyme-treated wheat straw (48). *Christensenellaceae* plays an important role in degrading fibre (49) and producing acetic and butyric acid (50). All the observations above were supported by former reports that feed processed by carbohydrase enzymes could stimulate growth of specific microbes (8, 9, 51). It can be speculated that the pretreatment of feed stuffs with carbohydrases causes the release of hydrolysis materials (presumably oligosaccharides), which promote the chemotactic response of specific bacteria, and stimulates their attachment to feed particles, thereby leading to growth of these microbes (9, 15-17).

Fibre degradation and SCFA production

Enzymatic and chemical treatment on RSM increased the amount of acetic acid, propionic acid, butyric acid, and thereby total SCFA production (Figure 6). This observation was consistent with the predicted functional profiles related to carbohydrate metabolism, where the relative abundance of fibre breakdown and fermentation enzymes and pathways increased (Figure 5). This finding was in accordance with previous studies that addition of pectolytic enzymes improved degradability of non-starch polysaccharides (NSP) of RSM *in vitro* (10) and in broilers (11, 12). Giraldo et al. also reported that supplementation of exogenous cellulase increased SCFA production (9). Previous research demonstrated that supplementation of carbohydrase on feed before feeding could increase microbial protein production, ruminal cellulolytic bacterial numbers, and ruminal fibrolytic activity (51). Thus, the findings above indicate that the enzymatic and chemical treatment on RSM could sufficiently open cell wall architecture (refer to Figure 2), to enable effective accessibility of NSP to bacterial degradation enzymes, and subsequently stimulate expression of microbial saccharolytic pathways.

No bindings of mAbs recognizing non-fucosylated XG were observed in lumen samples after the microbiota was fed with ALK or CON (Figure 7), which was unexpected as binding signals were seen in the substrates themselves prior to addition to SLIM (Figure 2). The hypothesis could be entertained that XG was immediately utilized by bacteria before our first sampling time point (after 30 minutes), or that the XG structures were unable to be recognized by the mAbs after supplementing them to lumen, due to entrapment by other molecules. Binding signals still existed at t24 for all mAbs that showed signal at the t0.5 time point, which indicated that these structures cannot be degraded any further or more fermentation time was needed. Glycome profiling showed that binding of mAbs recognizing each polysaccharide structure/epitope were dynamic during the 24 h fermentation period (Figure 7). It is not unlikely that certain polysaccharide structure, such as (hemi)cellulose and pectin, were exposed to microbes stage by stage, due to ever increasing degradation of the cell wall structures over time. For instance, bacteria should break down side-chain AG before they can utilize RG-I. However, this hypothesis should be validated in future study.

The increased in abundance of *Ruminococcaceae* NK4A214 group, *Ruminococcaceae* UCG-002, and *Ruminococcaceae* UCG-005 in enzymatic and chemical treated RSM groups showed negative correlations with XG, RG-I, and RG-I/AG (arabinogalactan side chains of RG-I), and positively correlated with AG-1, AG-2, and pectic backbone. These observations suggested that ALK, CELL, PECT1, and PECT2 stimulated these genera to utilize XG, RG-I, and arabinogalactan side chains of RG-I, and exposed AG-1, AG-2, and pectic backbone to other bacteria in the current study. The abundance of *Roseburia* and *Succinivibrionaceae* UCG-001 in enzymatic and chemical treated RSM groups had negative correlations with XG and AG-1, which suggested that the treatments on RSM stimulated these genera to degrade XG and AG-1. A possible model of action can be explained by the adhesion theory of Rumen Cellulolytic Bacteria (52), which supposes that the (in our case) enzymatic and chemical treatments on RSM stimulate attachments of microbes to specific polysaccharides structures (53), which lead the bacteria to degrade them. However, the mechanism of exogenous enzymes enhanced degradation of plant cell walls is complex (54), with many interrelated factors, and requires further studies. Moreover, degradation of fibres requires a plethora of

microbial enzymes as indicated for instance by the numerous PUL-loci needed by *Bacteroides thetaiotaomicron* to breakdown pectin (54).

Conclusion

The present study clearly demonstrated that both enzymatic and chemical pretreatment on RSM shifted its cell wall polysaccharide structure, subsequently altering microbial community composition and functional profile compared to untreated RSM, and eventually increased fibre degradability as evaluated by SCFA production. Furthermore, glycome profiling showed that the abundance of cell wall polysaccharides were dynamically changed during fermentation, and did not continuously decrease during the fermentation period as one might expect. Our findings that ALK, CELL, PECT1 and PECT2 increased fiber degradability in RSM could help guide feed additive strategies to improve efficiency and productivity in swine industry. The current study gave insight into how feed enzyme modulate microbial status, which provides good opportunity to develop novel carbohydrase, particularly in swine feed.

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Supplemental figures:

Figure S1. Community diversity represented by Shannon index at ASV level for samples from each time point (A) and treatment (B). ** $P < 0.01$.

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Figure S2. Principal coordinate analysis (PCoA) plot generated based on the calculated distances in weighted UniFrac matrix. Samples were grouped by shape and color in terms of treatment and time point they belonged to, respectively: CON (sphere), ALK (plus), PECT1 (triangle), PECT2 (diamond), CELL (square); a red-green-purple scale was used to indicate the fermentation time (red and purple depict start and end of the fermentation period).

Figure S3. Community diversity represented by Shannon index at ASV level for samples from before and after predigestion.

3

Chapter 4

Differently pre-treated rapeseed meals affect *in vitro* swine gut microbiota composition

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Abstract

The aim of the study was to investigate the effect of untreated and processed rapeseed meal (RSM) on fibre degradability by pig gut microbiota and the adaptation of the microbiota to the substrate, by using the Swine Large Intestine *in vitro* Model (SLIM). A standardized swine gut microbiota was fed for 48 h with pre-digested RSM which was processed enzymatically by a cellulase (CELL), two pectinases (PECT), or chemically by an alkaline (ALK) treatment. Amplicons of the V3-V4 region of the 16S rRNA gene were sequenced to evaluate the gut microbiota composition, whereas short chain fatty acids (SCFA) were measured to assess fibre degradation. Adaptive gPCA showed that CELL and ALK had larger effects on the microbiota composition than PECT1 and PECT2, and all substrates had larger effects than CON. The relative abundance of family Prevotellaceae was significantly higher in CELL treatment compared to others. Regardless of the treatments (including CON), the relative abundance of *Dorea*, *Allisonella*, and *FamilyXIIIUCG_001* (in the order of Clostridiales) were significantly increased after 24 h, and *Parabacteroides*, *Mogibacterium*, *Intestinimonas*, *Oscillibacter*, *RuminococcaceaeUCG_009*, *Acidaminococcus*, *Sutterella*, and *Citrobacter* were significantly higher in abundance at time point 48 compared to the earlier time points. *Prevotella* 9 had significant positive correlations with propionic and valeric acid, and *Mogibacterium* positively correlated with acetic and caproic acid. There was no significant difference in SCFA production between untreated and processed RSM. Overall, degradability in the processed RSM was not improved compared to CON during the RSM adaptation period. However, the significantly different microbes detected among treatments, and the bacteria considerably correlating with SCFA production might be important findings to determine strategies to shorten the fibre adaptation period of the microbiota, in order to increase feed efficiency in the animal, and particularly in pig production.

Introduction

Rapeseed meal (RSM) is not only an important alternative feed ingredient for protein-rich feeds (where mainly soybean meal is used as protein source), but RSM is also rich in non-starch polysaccharides (NSP) (1-3). However, a disadvantage of RSM is that a high proportion of cell wall polysaccharides cannot be utilized by endogenous enzymes from monogastric animals, and the NSP can affect the digestion of other nutrients by physical hindrance or physiological shift in the intestine (e.g. increasing digesta viscosity) (3). NSP can only be fermented in the swine large intestine by the gut microbiota, and research has shown that the pig gut microbiota needs an adaptation period to express its maximum enzymatic potential after a dietary change (4). However, most of the scientific research focused on how the fibres status affected nutrient digestibility or animal performance during the adaptation period (5, 6), and few monitored how the gut microbiota changed during this period (7).

Previous studies showed that a high-fibre rapeseed diet did not results in a significant increase in SCFA content in the chyme of RSM-fed pigs after a 3-week adaptation period (7, 8). However, SCFA-producing microbes, such as *Dialister*, *Shuttleworthia*, *Bulleidia*, *Coprococcus*, and *Lachnospira*, were detected more abundant in the colon of high-fibre rapeseed pigs compared to those in the control pigs (7). This might be because the 3-week adaptation period was too short to have a considerable change in the metabolic function of SCFA-producing microbes, or more likely, the SCFA were rapidly utilized by the intestinal epithelium of the host. Adaptation duration in pigs supplemented with 19.5 % palm kernel showed that 3 weeks of adaptation is needed, and a longer adaptation time is suggested as dietary palm kernel content of the diet increases (6). Huang et al. (2018) also observed that apparent total tract digestibility of acid detergent fibre significantly increased when the adaptation time increased from 7 to 28 days (6). Another report showed that microbial cellulase activity was only observed after a 6-week adaptation period when pigs were fed with different types of dietary fibre (8% sugar beet pulp, or 10% wheat bran) (9). Montoya et al. (2018) demonstrated that an optimum adaptation time also depends on the dietary level when growing pigs were fed with kiwi fruit (10). Thus, the optimal adaptation time should be evaluated case by case.

Treatment with carbohydrases, such as cellulase and pectinase, have been reported to improve the NSP degradation after a 4-week adaptation in both boilers and *in vitro* (3, 11). Thus, in the current study, RSM (predigested) was treated independently with two kinds of pectinases (PECT1 and PECT2), one cellulase (CELL), or alkaline (ALK), and afterwards the untreated and treated RSM preparations were fed to the recently developed Swine Large Intestine *in vitro* Model (SLIM) (12) for a 48 h period. SLIM was established on the basis of the human, computer-controlled, dynamic TNO *in vitro* model of the colon, TIM-2 (13), which accurately simulates the physiological conditions in the lumen of the human proximal colon, containing a gut microbiota of human origin. A unique design of the machine is that the model is equipped with a dialysate system, which prevents accumulation of microbial metabolites which would lead to the inhibition or death of microbes in the model when they would accumulate. Since all metabolites (i.e. SCFA) are collected, and basically a mass-balance can be made, this allows for real determination of the production of microbial metabolites such as SCFA, which is not possible *in vivo*.

In the current study, the effects of processed rapeseed meal on swine gut microbiota and SCFA production were investigated. We hypothesized that 1) the treatments on RSM will increase SCFA production (fibre degradability) during the fibre fermentation period compared to when the swine gut microbiota is fed with untreated RSM, and 2) the effects of processed RSM on the swine gut microbiota are different according to the treatments. The results of the current study give insight into how processed RSM affect pig gut microbiota during the fermentation period, which is important information to use to shorten the feed adaptation period and to improve feed efficiency.

Materials and methods

Substrate preparation

Rapeseed meal (*Brassica napus*, Cargill N.V., Antwerp, Belgium; 2011) was obtained from a commercial feed mill (Agrifirm B.V., Utrecht, the Netherlands). Preparation method I (predigesting RSM after carbohydrase or alkaline treatment; Figure 1): to 200 g of substrate 40 mL 10*gastric electrolyte concentrate solution (GES, 310 g sodium chloride, 110 g potassium chloride, 15 g calcium chloride di-hydrate, and 4840 g ultrapure water) and 360 mL ultrapure water were added. The pH was adjusted to 5.5 and then nothing (CON), ALK (6 M NaOH) or 10 mL of the following carbohydrases were added; CELL, Accellerase 1000 (Sigma-Aldrich, Missouri, United States); PECT1, Pectinex Ultra SP (Novozymes A/S, Bagsvaerd, Denmark); or PECT2, Multifect Pectinase (DuPont Industrial Biosciences, Genencor division, Rochester, NY). Enzyme preparations were incubated at 37 °C for 2 h, with occasional shaking (every 30 mins), while ALK was incubated overnight at 4 °C. Enzyme preparations were then heated at 100 °C for 5 min to inactive enzymes. Afterwards, for all five samples, 120 mL GES was added and the pH adjusted to 3 to continue with the gastric incubation according to the predigestion protocol as described elsewhere (14). After predigestion, the slurry was centrifuged (8.000 g, at 4 °C, for 20 minutes) and dialysis was performed for the supernatants. For dialysis, a dialysis membrane (Sureflux, Nipro Europe Group Companies, Mechelen, Belgium) was used with a peristaltic pump to remove small digestion products and water. After reduction of the total volume to ~450-500 mL, supernatant was mixed with pellet, and freeze-dried. Method II (predigesting RSM before carbohydrase or alkaline treatment; Figure 1): four batches of 200 g RSM were predigested as described before (14) and then dialyzed. Afterwards, 55 mL 10*GES was added, and pH adjusted to 5.5, after which 10 mL of CELL, PECT1, PECT2, or ALK treatment commenced, respectively. Enzyme preparations were incubated at 37 °C for 2 hours with occasional shaking (every 30 mins), and ALK was incubated overnight at 4 °C. Afterwards, enzyme preparations were heated at 100 °C for 5 min to inactive enzymes, and pH was neutralized to 6.5-7 with HCl or NaOH, and the samples were freeze-dried.

Figure 1. Schematic presentation of experimental setup for fibre fermentation in the Swine Large Intestine in vitro model (SLIM).

Fermentation in the Swine *in vitro* Large Intestinal Model (SLIM)

The setup of SLIM was as follows: a fully computer-controlled *in vitro* model based on TIM-2 (13) was used to mimic the swine large intestine (Figure S1). The pH (5.9) was controlled by continuous addition of 2 M sodium hydroxide. Standard ileal efflux medium of pigs (SIEMP) was used to simulate the materials entering the colon. The SIEMP, adapted from (15) and described in Long et al. (12), contained the following components (g/L): 74.6 maize starch, 9.0 xylan, 19.0 pectin, 9.0 amylopectin, 9.0 arabinogalactan, 9.0 arabinoxylan, 9.0 xyloglucan, 31.5 Tween 80, 43.7 casein, 0.7 ox-bile, 43.7 bactopecton, 4.7 K₂HPO₄·3H₂O, 0.009 FeSO₄·7H₂O, 8.4 NaCl, 0.8 CaCl₂·2H₂O, 0.7 MgSO₄·7H₂O, 0.05 bile, 0.02 haemin and 0.3 cysteine·HCl, plus 1.5 mL of a vitamin mixture containing (per litre): 1 mg menadione, 0.5 mg vitamin B12, 2 mg D-biotin, 10 mg pantothenate, 5 mg p-aminobenzoic, 4 mg thiamine and 5 mg nicotinamide acid. The pH was adjusted to 5.9. SIEMP only contains indigestible carbohydrates and hence did not require pre-digestion. Dialysis liquid contained (per litre): 2.5 g K₂HPO₄·3H₂O, 0.005 g FeSO₄·7H₂O, 4.5 g NaCl, 0.45 g CaCl₂·2H₂O, 0.05 g bile, 0.5 g MgSO₄·7H₂O and 0.4 g cysteine·HCl, plus 1 mL of the vitamin mixture. All medium components were purchased at Tritium Microbiology (Eindhoven, The Netherlands). The pig fecal inoculum was a standardized microbiota from growing pigs (48 pens with 6 pigs/pen, Hypor Libra x Hypor Maxter, Hendrix Genetics, Boxmeer, The Netherlands), consisting of pooled freshly collected feces from the floor, but only material from the top (so not touching the floor) was selected. Since this was not an intervention study, feces collection from the floor of the pens of the pigs did not require ethical approval in accordance with local/national guidelines.

In order to create a complete anaerobic environment, SLIM with 90 mL dialysate in each unit was flushed with gaseous nitrogen for at least 3 hours before incorporating the standardized microbiota (Figure S1). Thirty mL of the standardized microbiota was added to the system, making the total volume of each SLIM-unit 120 mL. The microbiota was adapted to the model with SIEMP for 16 hours. During the adaptation phase (Figure 1) SIEMP was administered at 60 mL d⁻¹ [corresponding to 7.5 g carbohydrate d⁻¹] (16) through the feeding syringe (Figure S1). At the end of the adaptation period, a 2-hour starvation period was performed (Figure 1), which was used to allow all the carbohydrates within SIEMP to be fermented by the microbiota and after this time point 0 was set. Afterwards, the 'fibre adaptation' period (48 hours) was performed, in which the microbiota was allowed to adapt to the test products (CON, CELL, PECT1, PECT2 and ALK). During this stage, carbohydrates in SIEMP were replaced with 7.5 grams of (treated) RSM which were added continuously in the model at a rate of 2.5 mL/h for 48 h (Figure 1), corresponding to 7.5 g carbohydrate d⁻¹.

Sample collection

Samples from lumen and spent dialysate were collected at time point 0, 24, and 48 h (t0, t24, and t48). They were snap-frozen in liquid nitrogen and stored until analyses. Lumen samples were used to analyze microbiota composition, and both lumen and dialysis samples were analyzed for short chain fatty acid (SCFA) concentrations.

Sequencing of V3-V4 region of the 16S rRNA gene

Microbial DNA extraction and sequencing of the V3-V4 region of the 16S rRNA gene were

performed by BaseClear B.V. (Leiden, The Netherlands). Briefly, genomic DNA extraction was performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, CA, USA) according to the manufacturer's instructions. Barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step PCR. 10-25 ng genomic DNA was used as template for the first PCR with a total volume of 50 µL using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers (17) appended with Illumina adaptor sequences. PCR products were purified (QIAquick PCR Purification Kit, Venlo, The Netherlands) and the size of the PCR products were checked on a Fragment analyzer (Advanced Analytical, Ankeny, US) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina, CA, USA). Subsequently, PCR products were purified, checked on a Fragment analyzer and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) and demultiplexed based on sample-specific barcodes. Raw sequencing data was submitted to the European Nucleotide Archive under the accession number: PRJEB38485.

Bioinformatics analysis

The demultiplexed raw sequences obtained from BaseClear were processed using QIIME2 pipeline (18). In short, reads were imported, quality filtered and dereplicated with q2-dada2 (19), after which dada2 was performed with paired-end reads and truncations parameters as follows: the first 10 base pairs were trimmed off and at position 280 base pairs the fragment was truncated in forward reads, and at position 240 base pairs for the reverse reads. The processed sequences were used for all the downstream analyses. Alpha-diversity (Shannon index) and beta diversity (weighted and unweighted UniFrac; (20, 21)) were analyzed by the q2-phylogeny plugin (<https://github.com/qiime2/q2-diversity>).

Adaptive generalized PCA, gPCA. Adaptive gPCA (22, 23) was performed on the ASV-tables generated in QIIME. This is a relatively new method which can obtain a low-dimensional representation of the samples in which the axes are interpretable at a fine phylogenetic scale, which can be used to compare the dissimilarities between samples more accurately than that of UniFrac. The mathematical algorithm for gPCA can be found in the literature (22). R packages biomformat, yaml, Biostrings, phyloseq, Hmisc, qiime2R, vegan, ggplot2, and adaptiveGPCA were used in this analysis.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, PICRUST2. PICRUST2 software (24) was used to predict microbial functional abundances based on marker gene sequences. KEGG database was used to predict the results.

Chemical analyses

Short-chain fatty acids analyses. Samples from lumen and dialysate were analyzed by Brightlabs (Venlo, The Netherlands) for determination of concentrations of SCFA (including the middle chain fatty acids valeric and caproic acid). Ion exclusion chromatography (IEC) was applied on an 883 Ion Chromatograph (IC; Metrohm, Herisau, Switzerland), using a Transgenomic IC Sep ICE-ION-300 column (30 cm length, 7.8 mm diameter and 7 µm particles) and a MetroSep RP2 Guard. The mobile phase consists of 1.5 mM aqueous

sulphuric acid. A column flow rate of 0.4 ml min⁻¹ was used. The temperature of the column was 65 °C. The organic acids were detected using suppressed conductivity detection. Samples were centrifuged (14,000 rpm, 10 min), and the clear supernatant was filtered through a 0.45 µm PFTE filter and diluted with mobile phase (for lumen 1:5, for dialysate 1:2). Ten microliters were loaded on the column by an autosampler 730 (Metrohm). Molecules were eluted according to their pKa.

Statistical methods

Kruskal-Wallis Rank Sum Test (one-way ANOVA on ranks) was applied to compare alpha diversities (Shannon index) among different RSM treatments and time points, and Wilcoxon Rank Sum Test was used for pairwise comparison in R version 3.5.3 (<https://www.r-project.org/>). Bonferroni was used to correct P-values. Permutational multivariate analysis of variance (PERMANOVA; (25)) was performed to test the significance of beta diversity (weighted and unweighted UniFrac) between non-processed and processed RSM in QIIME2. The results were visualized in R. Linear discriminant analysis effect size [LEfSe] (26) was used to find biomarkers between groups using relative abundances from the feature tables generated in QIIME at genus level.

Pearson correlations between continuous meta-variables (SCFA production) and taxonomic variables were calculated and visualized in R. Parameters were set as follows: Missing values for meta-variables were handled as NO imputation (replacing missing data with substituted); zeros were kept for the calculation of correlation; a minimum relative abundance of 0.1 % was considered for calculation; a minimum of 4 pairs of observations were required for calculation of correlations.

T-tests were conducted to compare SCFA production among CON, ALK, PECT1, PECT2, and CELL in the built-in R package.

Results

RSM with different treatment methods differentially changed the microbial phylogenetical relationship during the fibre fermentation period

There were no significant difference in α -diversity (Shannon Index) between non-processed RSM (CON) and processed RSM (ALK, PECT1, PECT2, and CELL) (Figure 2A). Alpha-diversity was significantly decreased during the fibre fermentation period (Figure 2B), significant at 72h. Unweighted UniFrac shows that CON had a significant different microbial community compared to ALK, PECT1, PECT2, and CELL while there were no significant difference between non-processed and processed RSM in terms of weighted UniFrac (Figure S2). The biplot of unweighted UniFrac (Figure S2) showed that genus *Prevotella* 9 was mainly associated with t0, while *Ruminococcaceae gauvreauii* group, *Eubacterium nodatum* group, *Succiniclasticum*, *Mogibacterium*, *Olsenella*, *Prevotellaceae NK3B31* group *Bacteroides S24-7* group *Rikenellaceae RC9* gut group, and *Ruminococcaceae UCG-002* were associated with t24 and t48. Afterwards, adaptive gPCA (23) was used to identify the dissimilarities among samples. The results show that the starting points (t0) of all the treatments were approximately located in the same spot of the principle plane, and the later time points lay along the first adaptive gPCA axis (Figure 3A). After 48 hours adaptation, the microbiota composition changed considerably in the processed RSM groups compared to the start time points, whereas CON only showed a slight difference between t48 and t0 (Figure 3B).

Figure 2. Community α -diversity represented by Shannon index from each treatment (A) and time point (B). The treatments are non-processed RSM (CON) and RSM processed by Accellerase 1000 (CELL), Pectinex Ultra SP (PECT1), Multifect Pectinase (PECT2), or 6M NaOH (ALK).

Figure 3. Two views of results from adaptive gPCA. (A) shows the sample scores from gPCA plotted on the first two axes. (B) sample scores have been centered by treatments so as to better show the within-treatment variation, and the centered scores along the first axis are displayed over time.

Alpha-diversity (Shannon index) and β -diversity (weighted and unweighted UniFrac) showed that there were no significant differences between microbiota fed with modified RSM before or after dialysis (data not shown). These indicated that predigesting before or after modifying RSM by ALK or enzymes did not differentially affect microbiome composition. Our previous study showed that predigesting before or after processing RSM had little effect on their monosaccharide constitution (Long et al. manuscript2 submitted). Therefore, they were treated as duplicates in the following microbial relative abundance analysis.

Fermentation time had larger effect on the microbiota composition during the fibre fermentation period than the treatments on RSM did

Figure 4A shows at the phylum-level that the relative abundances of Bacteroidetes in response to ALK, CON, PECT1, PECT2, and CELL were quite similar to each other at t0, whereas they continuously decreased with ALK, PECT1, and CELL feeding at both t24 and t48 compared to t0, while feeding CON and PECT2 decreased the relative abundances of Bacteroidetes at t24, which slightly increased again at t48. The relative abundance of Firmicutes in ALK, CON, PECT1, and PECT2 was quite stable during the fibre fermentation period, while it continuously increased in CELL. The relative abundances of Actinobacteria in ALK, PECT1, and PECT2 increased, whereas those of CON and CELL were stable during the fibre fermentation period. ALK decreased the relative abundance of Proteobacteria, while the others treatments increased this phylum during the fibre fermentation period.

Figure 4. Relative abundances of microbial phyla (A) and families (B) in pig microbiomes fed with non-processed RSM (CON) or RSM processed by Accellerase 1000 (CELL), Pectinex Ultra SP (PECT1), Multifect Pectinase (PECT2), or 6M NaOH (ALK) at different time points (0 h, 24 h, and 48 h).

Figure 4B shows at the family level that the different substrates had an effect on relative abundance at the family level, and the top six most abundant families are summarized below. ALK, PECT2, and CELL feeding decreased the relative abundance of Prevotellaceae, Ruminococcaceae, and Muribaculaceae. Lachnospiraceae was stable, while Atopobiaceae and Acidaminococcaceae increased upon feeding ALK. Lachnospiraceae decreased, Atopobiaceae increased, and Acidaminococcaceae was stable upon feeding PECT2. Lachnospiraceae increased, Atopobiaceae was stable, and Acidaminococcaceae decreased with CELL treatment. CON and PECT1 feeding decreased the relative abundance of Prevotellaceae, Lachnospiraceae, and Muribaculaceae, and stabilized Ruminococcaceae and Acidaminococcaceae. The numbers of Atopobiaceae decreased with CON, while it increased with PECT1.

Figure 6. PCA plots of the relative abundances of Enzyme Classification numbers (A) and pathways (B) from microbiotas fed with CELL, CON, PECT1, PECT2, and ALK at different time points (0, 24, 48 h).

In this study we focused on carbohydrate metabolism related microbial functions. Figure 7 shows that 8 pathways were significantly increased after the fibre adaptation period, and they were D-galactarate degradation I, D-glucarate degradation I, glyoxylate cycle, pyruvate fermentation to acetone, TCA cycle IV (2-oxoglutarate decarboxylase), superpathway of “D-glucarate and D-galactarate degradation”, superpathway of “glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass”, and superpathway of “glyoxylate bypass and TCA”. On the other hand, 4 pathways were significantly decreased after the fibre adaptation period, which were D-galacturonate degradation I, lactose and galactose degradation I, mannan degradation, and pyruvate fermentation to propanoate I. Additional significant increased other pathways and all enzymes are displayed in Table S1 and S2, respectively.

Figure 7 Significantly different metagenomic functions in relative abundance among different time points.

There were no significantly different pathways between treatments and CON.

The cumulative amount of SCFA continuously increased during the fibre fermentation period

Figure 8A shows the average cumulative amount of total, individual SCFA (acetic, propionic, butyric, and including the middle chain fatty acids valeric and caproic acid) at t24 and t48. When mentioning total SCFA or SCFA production, the middle chain fatty acids valeric and caproic acid, were included in the current context. Production of total and individual SCFA constantly increased after 24 and 48 h of fermentation. As generally observed, more acetic acid was produced compared to the amount of propionic and butyric acid, which were similar to each other. There were no significant difference between the first 24 h (t24) and second 24 h (t48) in SCFA production, except for propionic acid production, where the amount of propionic acid production at t24 was significant higher than that at t48 ($P < 0.05$), independent of substrate (Figure 8B). The first 24 h of fibre adaptation resulted in an increase of 7.0, 7.4, 7.5, 6.3 and 6.6 mmol total SCFA/g substrate in response to ALK, CELL, CON, PECT1, and PECT2, respectively. This slightly increased during the second 24 h of fermentation, to 7.1, 8.2, 7.9, and 7.0 mmol total SCFA/g substrate in response to ALK, CELL, CON, and PECT1 feeding, respectively. Only for PECT2 the SCFA production slightly decreased during the second 24 h of fermentation compared to the first 24 h (5.7 mmol SCFA/g substrate).

Figure 8. Average cumulative short chain fatty acid (SCFA) production in mmol (A) and SCFA increase in mmol/g substrate/24h (B) for non-processed RSM (CON) and RSM processed by Accellerase 1000 (CELL), Pectinex Ultra SP (PECT1), Multifect Pectinase (PECT2), or 6M NaOH (ALK). The values at time point 0 were artificially set to 0 to allow determination of cumulative production. (C) correlation between SCFA production and core genera, * $p < 0.05$; blue: positive correlation; red: negative correlation. Core genera: the table was normalized via division by the sum of sequences in a given sample and multiplied by the minimum sum across all samples. Relative abundances were filtered as follows: values below a relative abundance threshold of 0.01% were not taken into account; taxa with a median relative abundance < 1 % in all groups were not considered for statistical analysis.

There were no significant differences in SCFA production between unprocessed and processed RSM during the fibre fermentation period.

Correlations between the relative abundance at genus level and SCFA production were evaluated (Figure 8C). *Prevotella 9* ($P_{\text{propionic acid}} < 0.01$, $P_{\text{valeric acid}} < 0.01$) and uncultured *Porphyromonadaceae bacterium* ($P_{\text{propionic acid}} = 0.03$, $P_{\text{valeric acid}} < 0.01$) had significant positive correlations with both propionic and valeric acid, while *Mogibacterium* ($P_{\text{propionic acid}} < 0.01$, $P_{\text{valeric acid}} < 0.01$) and *[Eubacterium] nodatum* group ($P_{\text{propionic acid}} = 0.02$, $P_{\text{valeric acid}} < 0.01$) had negative correlation with these acids. Caproic acid significantly negatively correlated with *Prevotella 9* ($P < 0.01$), *Ruminococcus 2* ($P = 0.03$) and uncultured

Porphyromonadaceae bacterium ($P = 0.01$), whereas *Mogibacterium* ($P_{\text{acetic acid}} = 0.01$, $P_{\text{caproic acid}} = 0.03$) and [*Eubacterium*] *nodatum* group ($P_{\text{acetic acid}} = 0.01$, $P_{\text{caproic acid}} = 0.03$) positively correlated with acetic and caproic acid. An unclassified genus from Prevotellaceae ($P < 0.01$) had positive correlation with valeric acid, while *Acetitomaculum* ($P < 0.01$) negatively correlated with it.

Discussion

The pig gut microbiota needs an adaptation period to express its maximum enzymatic potential after a change of diet (4). In the current study, treatment of RSM was performed prior to ingestion to decrease this adaptation period, but no significant difference was detected in SCFA production among non-processed (CON) and processed RSM (ALK, PECT1, PECT2, and CELL) fermentation during the 48 h fibre fermentation period in the swine *in vitro* large intestine model (SLIM). However, when another 24 h fermentation period was performed after this 48 h ‘fibre adaptation’ period, considerable changes were found in the amount of SCFA production in response to different treatments (Long et al. manuscript submitted). Previous studies show that treatments (ALK, PECT1 and PECT2) on RSM did improve the NSP degradation after 4-week adaptation in boilers and pigs (3, 27). Thus, it is important to know how the microbiota changes during the fibre adaptation period. The current study showed that essentially a 48 h period was sufficient to increase the pathways, involved in carbohydrate fermentation.

Alpha-diversity significantly decreased after 48 h RSM fermentation, while there were no significant changes between treatments and CON during the fibre adaptation period. This may due to the selection of particular microbes to utilize RSM regardless of the treatment, since RSM was the major nutrient in all cases, compared to the microbial adaptation period (16 h SIEMP adaptation), and the different treatments applied only led to changes in cell wall structures in RSM (2). In previous studies, it has been shown that RSM increased the relative abundance of *Dorea* and *Lactobacillus* in chickens (28, 29). *Lachnospira*, *Coproccoccus*, *Bulleidia*, and *Shuttleworthia* are increased by RSM after three weeks adaptation in pigs (7). In the current study, a large number of genera (24 genera) significantly decreased after the fibre fermentation period, and only three and eight genera were considerably increased after adapting to the substrate for 24 h and 48 h, respectively. The decreased taxa may not utilize RSM well, while RSM may be beneficial for the increased taxa. The significantly increased genera observed in the current study are *Dorea*, *Allisonella*, *FamilyXIIIUCG_001* (in the order of Clostridiales), *Parabacteroides*, *Mogibacterium*, *Intestinimonas*, *Oscillibacter*, *Sutterella*, *Citrobacter*, *RuminococcaceaeUCG_009*, and *Acidaminococcus*. No overlapping genera were observed between the current study and research of Umu et al. (7), which might be because of a different pig breed. On the other hand, *Dorea* was found significantly increased by RSM in both the current study and research of Long et al., where six weeks of adaptation period was performed in laying hens (28).

Disappearance of some low abundance microbes were observed in either processed or non-processed RSM treatment since unweighted UniFrac (only considering presence or absence) was found significantly different ($P = 0.02$), while there was no difference in terms of weighted UniFrac (accounting also for abundance of observed organisms) among the treatments. This indicated that the treatments applied on RSM indeed have an effect on the microbiota composition, but that the short fibre fermentation period used may not yet have led to significant changes. In another study, we showed however that weighted UniFrac was

observed to be significantly different among the treatments when a single shot of the fibres was introduced after this initial 48 h (Long et al. manuscript submitted). Zooming in to individual taxa, at the family level, Prevotellaceae significantly increased with CELL. Prevotellaceae is an important family, which is involved in degrading hemicellulose (30). We also observed that genera (e.g. *Prevotella* 9) from Prevotellaceae significantly positively correlated with SCFA production (Figure 7). To demonstrate the dynamic changes in swine microbiota composition in response to differently pretreated RSM, lumen samples from each time point were analyzed. The most dominant phyla were Firmicutes, Bacteroidetes, and Actinobacteria at t0, regardless of treatment (Figure 4). This was in line with previous studies (31-34), which indicated that core microbes existed. The relative abundance of Bacteroidetes decreased after supplementing with ALK, PECT1, and PECT2, whereas that of Actinobacteria increased (Figure 4). These observations implied that supplementation of ALK, PECT1, and PECT2 stimulated the growth of microbes from phylum Actinobacteria and inhibited the growth of bacteria from phylum Bacteroidetes. Actinobacteria has been reported to efficiently utilize (hemi)cellulose (35-37). Firmicutes and Proteobacteria were stimulated after supplementing with CELL (Figure 4). Firmicutes is a phylum that consists of a group of dominant butyrate-producing bacteria (38, 39), which play an important role in carbohydrate degradation. Research showed that the less abundant phylum, Proteobacteria, has significant functional variability in human gut microbiota (40). Zooming in to family level, ALK, PECT1, and PECT2 stimulated the growth of Atopobiaceae (phylum Actinobacteria); CELL benefited the growth of Lachnospiraceae (phylum Firmicutes). The observations above suggested these microorganisms that were stimulated by these treatments play roles in the fibre degradation. Altogether, this indicated that fibre adaptation period competitively excluded low abundant microbes and created a better ecological niche for the growth of RSM-utilization (fibre fermentation) members of the microbiota.

CELL and ALK have larger effect on pig gut microbiota compared to other treatments, since their within-treatment distances between t0 and t48 were larger than those of CON, PECT1, and PECT2 (Figure 3). Our other study, with the fibre shot after 48 h adaptation, showed also that more genera were observed to be significantly changed particularly upon feeding CELL and ALK than with PECT1 and PECT2, when compared to CON (Long et al. manuscript submitted). The primary cell wall of RSM contains a backbone of cellulose microfibrils, which are interlinked with xyloglucan via hydrogen bonds forming a stiff network (41). Pectins are linked to each other and cross-linked with hemicellulose and cellulose (42). CELL might have relative easy access to target cellulose in RSM, whereas PECT1 and PECT2 could have less opportunity to access pectins, supposedly hindered by cellulose microfibrils. Cell wall structures processed by CELL would lead to utilization of the fibres by microbes, and increase their abundances. Alkaline pretreatment of RSM breaks the alkali-labile bonds, which are known to hinder the complete fermentation of NSP in swine, and improved NSP utilization in feed (2). However, also PECT1 and PECT2 affected the swine gut microbiota in these experiments, since gPCA showed that their distances between time point 0 and 48 h were larger than that of CON. A previous report showed that a cocktail of PECT1 and PECT2 improved degradability of non-starch polysaccharides of RSM in broilers (3). Treatments on RSM, either with carbohydrases or alkaline, did not improve SCFA production during the fibre fermentation period compared to non-processed RSM. This indicated that the processing applied to RSM could not improve their degradability compared to CON, in other words, they might not increase the feed efficiency during this period *in vivo*. It might be the

fibre adaptation period was not enough to express its maximum enzymatic potential (4), although as mentioned earlier within 48 h the pathways involved in carbohydrate fermentation as increased (Figure 7A). Moreover, our own experiments with the fibre shot after 48 h showed that more pathways related to fibre fermentation were predicted to be upregulated (Long et al. manuscript submitted). PCA plots of Enzyme Classification numbers and pathways also show that samples from t0 clustered together, but separately from samples from t24 and t48. This also indicated that microbial function between t0 and later time points was modulated, and geared towards degradation of these new substrates. Previous studies showed that a high-fibre rapeseed diet did not results in a significant increase in SCFA content in the chyme of RSM-fed pigs after a 3-week adaptation period (7, 8), which seemed to indicate that the adaptation period was not long enough. Thus, strategies should be considered to shorten the fibre adaptation period, in order to increase feed efficiency.

In conclusion, the current study demonstrated that microbiota composition was significantly affected by both RSM (processed or not) and adaptation time. Unweighted UniFrac showed that microbial community composition was significantly separated between processed RSM and CON, and CELL and ALK in general changed the microbiota composition more than PECT1 and PECT2 did. Carbohydrate metabolism related microbial functions were significantly increased after the fibre fermentation period. However, degradability of the processed RSM was not improved compared to CON during the fibre fermentation period, as assessed by SCFA production, which indicated a relative long adaptation period is needed after a diet change to RSM for swine microbiota. Thus, the significantly different microbes detected between treatments, and the bacteria considerably correlating with SCFA production might be an important finding to design specific strategies to shorten the fibre adaptation period, in order to increase feed efficiency in animals, and particularly in pig production.

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Supplemental figures:

Figure S1 SLIM system. A. peristaltic compartments; B. pH-electrode; C. alkali pump; D. dialysis liquid circuit with hollow fibers; E. level-sensor; F. N₂ gas inlet; G. sampling port; H. gas outlet; I. 'ileum efflux' container, or, food syringe; J. temperature sensor.

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Figure S2. Principle coordinate analysis (PCoA) plots generated based on the calculated distances in unweighted (A) and weighted (B) UniFrac matrices. C: Biplot of unweighted UniFrac. Samples were grouped by shape and color in terms of treatment and time point they belonged to, respectively: non-modified RSM (CON) star, RSM modified by 6 M NaOH (ALK) sphere, RSM modified by Pectinex Ultra SP (PECT1) square, RSM modified by Multifect Pectinase (PECT2), triangle, and RSM modified by Accellerase 1000 (CELL), ring; time point 0 h (red), 24 h (blue), 48 h (orange). The trajectories started from time point 0 h, and ended with time point 48 h.

Figure S3. LefSe results of pig microbiota fed with (A) CELL and CON, and (B) at time point 0, 24, and 48 h.

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Chapter 5

Cellulase and alkaline treatment improve intestinal microbial degradation of recalcitrant fibres of rapeseed meal in pigs

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ABSTRACT The aim of current study was to investigate whether degradation of rapeseed meal (RSM) by a swine gut microbiota consortium was improved by modifying RSM by treatment with cellulase (CELL), two pectinases (PECT), or alkaline (ALK) compared to untreated RSM and to assess whether microbiota composition and activity changed. The predicted relative abundances of carbohydrate digestion and absorption, glycolysis, pentose phosphate pathway, and pyruvate metabolism were significantly increased upon CELL and ALK feeding, and CELL and ALK also exhibited increased total short-chain fatty acid (SCFA) production compared to CON. *Megasphaera*, *Prevotella*, and *Desulfovibrio* were significantly positively correlated with SCFA production. Findings were validated in ileal cannulated pigs, which showed that CELL and ALK increased fibre degradation of RSM. In conclusion, CELL and ALK rather than PECT1 or PECT2 increased fibre degradation in RSM, and this information could guide feed additive strategies to improve efficiency and productivity in the swine industry.

Introduction

The European Union (EU) is highly dependent on imports of protein-rich animal feed ingredients (70 %). This percentage is even higher when the focus is on soya beans alone, where the EU imports 95 % of its demand or on average 36.1 million tons of soybean equivalent on a yearly basis ¹. Of these, 9 million tons of soybean meal are annually used in pig production. In order to have a more sustainable supply of responsible protein-rich feed ingredients, the European livestock sector needs an alternative local protein feed ingredient to fill the “protein gap”.

Rapeseed meal (RSM), a byproduct from rapeseed oil production, is not only a suitable protein source for swine feed but also a potentially energy source. RSM contains a high amount of cell wall polysaccharides, even higher when compared to soybean meal commonly used in the feed industry. Non-starch polysaccharides (NSP) constitute 20 to 40 % of RSM ²⁻³, which are represented by pectic polysaccharides (homogalacturonan, rhamnogalacturonan, arabinogalactan and arabinan), cellulose, and hemicelluloses (xyloglucan, galactomannan, and glucuronoxylan) ⁴⁻⁵. A drawback of RSM is that the complex cell wall polysaccharides cannot be utilized by endogenous enzymes from monogastric animals, and also can only partly be fermented by the microbial community in the gastrointestinal tract (GIT). Reports show that NSP can only be degraded for 3 to 6 % in chicken ^{3, 6-7}, and around 58 to 68 % in pigs, which is rather low compared to other NSP-rich feed ingredients, such as sugar beet pulp (approximately 85 % of NSP is degraded by pigs) ⁸. Thus, pre-treatment of RSM is needed to improve its digestibility and fermentability. Usage of carbohydrases, e.g. β -glucanases, xylanases, cellulase and/or pectinase, is common in poultry feed, however less feed enzymes are used in pig diets with respect to increasing fibre degradation.

Meanwhile, the intestinal microbiota plays a critical role in host nutrition, health, performance and quality of meat products, since the microbiota in the GIT can degrade the undigested substrates, and create SCFA and oligosaccharides from cell wall NSP, acting as an additional energy source and having a potential prebiotic effects, respectively ⁹. The chemical composition and structure of the substrates largely determine the (changes in) microbial composition of the bacterial community in the GIT, as microbes differ in their substrate preferences (degradation capacity) and growth requirements ¹⁰⁻¹¹. As a result, microbial composition and metabolic function are very much dependent on biochemical conditions of digesta. Torok and colleagues ¹² investigated changes in gut microbial population in response to the supplementation of an NSP-degrading enzyme (containing β -glucanase, xylanase, and protease activities) in a barley-based diet in chickens, and the results showed that microbial composition revealed distinct clusters correlating with un-supplemented and enzyme supplemented birds. Supplementation of carbohydrases has been shown to modulate gut microbiota in a limited number of studies, both in animal and *in vitro* models ¹³⁻¹⁵.

In the current study, RSM (predigested with digestive enzymes) was treated independently with two kinds of pectinases (PECT1 and PECT2), one cellulase (CELL), or alkaline (ALK), and afterwards the untreated and treated RSM preparations were fermented in the Swine Large Intestine *in vitro* Model (SLIM) ¹⁶. and *in vivo* (in ileal cannulated pigs). We hypothesized that 1) carbohydrase increases NSP degradability of RSM, and 2) feed

enzymes-treated RSM differentially affects the pig gut microbiota composition and through this affects the predicted microbial functional profile and potential energy yield of the substrate. The 16S rRNA gene sequencing technology was used to monitor the microbial communities. The results of the current study provide insight into how carbohydrases affect swine gut microbiota, which is important information to exploit (new) feed enzymes.

Materials and methods

Substrate preparation

Rapeseed meal (*Brassica napus*, Cargill N.V., Antwerp, Belgium) was obtained from a commercial feed mill (Agrifirm B.V., Utrecht, the Netherlands). Preparation method I (predigestion of RSM after carbohydrase or alkaline treatment) [Figure 1] was as follows: to 200 g of RSM 40 ml 10*gastric electrolyte concentrate solution (GES, 310 g sodium chloride, 110 g potassium chloride, 15 g calcium chloride di-hydrate, and 4840 g ultrapure water) and 360 g/mL ultrapure water were added. The pH was adjusted to 5.5 and then nothing (CON), 10 mL of alkaline (ALK, 6 M NaOH), or the following carbohydrases were added: CELL (Accellerase 1000, Sigma-Aldrich, Missouri, United States), PECT1 (Pectinex Ultra SP, Novozymes A/S, Bagsvaerd, Denmark), or PECT2 (Multifect Pectinase, DuPont Industrial Biosciences, Genencor division, Rochester, NY). Enzyme/substrate mixtures were incubate at 37 °C for 2 h, with occasional shaking (every 30 mins), while ALK was incubated overnight at 4 °C. Enzyme/substrate mixtures were then heated at 100 °C for 5 min to inactive enzymes. For all treatments, pH was neutralized to 6.5-7 with HCl or NaOH. Afterwards, for all five samples, 120 mL GES was added and pH adjusted to 3 to continue with the gastric incubation according to the predigestion protocol as described elsewhere ¹⁷. After predigestion, the slurry was centrifuged (8,000 g, 4 °C, 20 min) and dialysis was performed for the supernatants. For dialysis, a dialyzer (Sureflux, Nipro Europe Group Companies, Mechelen, Belgium) was used with a peristaltic pump to remove small digestion products and water. After reduction of the total volume to ~450-500 mL, supernatant was mixed with the pellet, and freeze-dried. For method II (digestion of RSM before carbohydrase or alkaline treatment) [Figure 1], four quantities of 200 g RSM were predigested as described above and then dialyzed. Afterwards, 55 mL 10*GES was added, and pH adjusted to 5.5, after which 10 mL of CELL, PECT1, PECT2, or ALK treatment commenced, respectively. Enzyme/substrate mixtures were incubate at 37 °C for 2 hours with occasional shaking (every 30 mins), and ALK was incubated overnight at 4 °C. Afterwards, enzyme/substrate mixtures were heated at 100 °C for 5 min to inactive enzymes, and pH was neutralized to 6.5-7 with HCl or NaOH. Samples from both method I and method II were subsequently freeze-dried. Samples are differentiated by the suffix _B (for before) or _A (for after) (e.g. PECT1_A) for carbohydrase- or ALK-treatment prior to and after digestion, respectively.

Figure 1. Schematic of experimental design.

Fermentation in The Swine *in vitro* Large Intestinal Model (SLIM)

The setup of SLIM has been described before (Long et al., 2020). Briefly, a fully computer-control *in vitro* model was used to mimic the swine large intestine. The pH (5.9) was controlled by the addition of 2 M sodium hydroxide. Standard ileal efflux medium of pigs (SIEMP) was used to simulate the materials entering the colon¹⁶. SIEMP and dialysate solution are described in detail in Long et al.¹⁶. Briefly, The SIEMP, slightly modified from¹⁸ and described in¹⁹ contained the following components (g/L): 74.6 maize starch, 9.0 xylan, 9.0 pectin, 9.0 amylopectin, 9.0 arabinogalactan, 9.0 arabinoxylan, 9.0 xyloglucan, 31.5 Tween 80, 43.7 casein, 0.7 ox-bile, 43.7 bactopecton, 4.7 K₂HPO₄·3H₂O, 0.009 FeSO₄·7H₂O, 8.4 NaCl, 0.8 CaCl₂·2H₂O, 0.7 MgSO₄·7H₂O, 0.05 bile, 0.02 haemin and 0.3 cysteine-HCl, plus 1.5 mL of a vitamin mixture containing (per litre): 1 mg menadione, 0.5 mg vitamin B12, 2 mg D-biotin, 10 mg pantothenate, 5 mg p-aminobenzoic, 4 mg thiamine and 5 mg nicotinamide acid. The pH was adjusted to 5.9. Dialysis liquid contained (per litre): 2.5 g K₂HPO₄·3H₂O, 0.005 g FeSO₄·7H₂O, 4.5 g NaCl, 0.45 g CaCl₂·2H₂O, 0.05 g bile, 0.5 g MgSO₄·7H₂O and 0.4 g cysteine-HCl, plus 1 mL of the vitamin mixture. All medium components were purchased at Tritium Microbiology (Eindhoven, The Netherlands). The pig fecal inoculum was a standardized microbiota from growing pigs collected from the floor (48 pens with 6 pigs/pen, Hypor Libra x Hypor Maxter, Hendrix Genetics, Boxmeer, The Netherlands), but only fresh feces and from the top (not touching the floor) was selected. Feces was pooled and mixed with dialysate as described before¹⁶.

In order to create a complete anaerobic environment, SLIM with 90 mL dialysate in each of the 4 individual units was flushed with gaseous nitrogen for at least 3 hours before incorporating the standardized microbiota. Thirty mL of the standardized microbiota was added to each SLIM-unit, making the total volume 120 mL. Figure 1 shows the experimental set-up for fibre addition to SLIM. The microbiota was adapted to the model with SIEMP for 16 hours. During the adaptation phase, SIEMP was added into each SLIM-unit at a rate of 2.5 mL/h through the feeding syringe. At the end of the adaptation period, a 2-hour starving

period was performed, which was used to allow all the carbohydrates within SIEMP to be fermented by the microbiota. Afterwards, the fibre adjustment period (48 hours) was performed, in which the microbiota was allowed to adapt to the test products (CON, CELL, PECT1, PECT2 and ALK-treated RSM). During this stage, carbohydrates in SIEMP were replaced with 7.5 grams/day of (treated) RSM which were added continuously in the model at a rate of 2.5 mL/h. At the end of the 48h adaptation period, a shot of 5 grams of the different RSMs was given to the system at time point 48 hours.

***In vivo* fermentation in growing pigs by means of the mobile nylon bag technique**

The mobile nylon bag technique (MNBT) studies were carried out at the Animal Nutrition Group of Wageningen University & Research in Wageningen, The Netherlands. Two pigs (TN 70, Topigs Norsvin) with initial body weight of 28 ± 6.8 kg were fitted with a simple T-cannula at the distal ileum²⁰ for the insertion of nylon bags. After surgery, the pigs were individually housed on tenderfeet floors with small openings. They were fed their diet as mash. All experimental procedures were approved by the local institution for animal welfare (IVD) of Wageningen University & Research.

The MNBT studies included the nine feedstuffs which are described above (CON, 4 RSM substrates treated with carbohydrases or ALK before digestion, and 4 RSM substrates treated with carbohydrases or ALK after digestion). The procedures were slightly modified from previous research²¹. Samples of 0.3 to 0.5 g of each feedstuff were ground and filled into a nylon cloth (bag size: 25 mm X 40 mm, pore size 48 µm, Sefar Nitex, Heiden Swiss, 03-37/24), and afterwards sealed by means of a heat sealer. Eight bags per feedstuff were prepared, 4 bags for 2 pigs. The bags were inserted in the distal ileum through the cannula divided over 10 days; two bags at a time with two or three insertion moments at 15 mins intervals per day. Some bags were not collected and these replicates were repeated. The average collection time was 126.1 mins (range from 42.3 mins to 175.5 mins). In total, 64 bags were retrieved from feces and directly frozen at -20 °C before transporting to the laboratory. Bags were subsequently cleaned from adherent feces using ultrapure water and thereafter immediately freeze-dried.

Sample collection

***in vitro* SLIM**

Lumen samples from time point 48 h (just before the shot) were analyzed for constituent monosaccharide composition, molecular weight distribution, and oligosaccharide profiling. Samples from lumen and spent dialysate were collected at time point 48.5, 49, 50, 52, 54, 56, and 72 h. They were snap-frozen in liquid nitrogen and stored until analyses. Lumen samples were used to analyze microbiota composition, constituent monosaccharide composition, molecular weight distribution, and oligosaccharide profiling, while both lumen and dialysis samples were analyzed for SCFA concentrations.

MNBT study

Samples from MNBT studies were pooled together according to treatment, and used to analyze constituent monosaccharide composition.

16S rRNA gene sequencing

Microbial DNA extraction and sequencing of the V3-V4 region of the 16S rRNA gene were performed by BaseClear B.V. (Leiden, The Netherlands). Briefly, genomic DNA extraction from a single sample at each time point was performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, California, US) according to the manufacturer's instructions. Barcoded amplicons from the V3-V4 region of 16S rRNA genes were generated using a 2-step PCR. 10-25 ng genomic DNA was used as template for the first PCR with a total volume of 50 µL using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers²² appended with Illumina adaptor sequences. PCR products were purified (QIAquick PCR Purification Kit, Qiagen, Venlo, The Netherlands) and the size of the PCR products were checked on a Fragment analyzer (Advanced Analytical, Ankeny, US) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina, California, US). Subsequently, PCR products were purified, checked on a Fragment analyzer and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2x) 300 bp protocol and indexing by Baseclear B.V. (Leiden, The Netherlands). The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes. Raw sequencing data was submitted to the European Nucleotide Archive under the accession number: PRJEB36980.

Bioinformatics analysis

The demultiplexed raw sequences obtained from BaseClear were processed using QIIME2 pipeline²³. In short, reads were imported and quality filtered and dereplicated with q2-dada2²⁴. Next, dada2 was performed with paired-end reads and truncation parameters were as follows: the first 10 base pairs were trimmed off and at position 280 base pairs the fragment was truncated in forward reads, and at position 240 base pairs for the reverse reads. The processed sequences were used for all the downstream analyses. Alpha-diversity (Shannon index) and beta diversity (weighted and unweighted UniFrac) were analyzed by the q2-phylogeny plugin (<https://github.com/qiime2/q2-diversity>). All scripts used in the current analysis were deposited in Supplemental file 1: R_Markdown.html.

Random Forest. The Random Forest supervised machine learning algorithm was performed to predict treatments and timepoints from microbiome composition. The predictive models were built in R using the “caret” package. Specifically, samples were divided into training (more than 60 % of the total samples) and test sets. Once the data were split, the function ‘train’ was used to fit the random forest model. Afterward, class labels on the test set were predicted by using the function ‘predict’, and compared to the real class labels. To interpret random forest results, proximity plots were produced in R. To understand more about the random forest model, the amplicon sequence variant (ASV) with the most influence in the random forest prediction was identified. All the analyses were done in R version 3.5.3 program, and the following packages were used: bioformat, yaml, Biostrings, phyloseq, Hmisc, qiime2R, vegan, ggplot2, tidyverse, caret, and randomForest.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, PICRUSt2. The PICRUSt2 software²⁵ was used to predict microbial functional abundances based on marker gene sequences. KEGG database was used to predict the results.

Chemical analyses

Short-chain fatty acids. Samples from lumen and dialysate were analyzed by Brightlabs (Venlo, The Netherlands) for determination of concentrations of SCFA. Ion exclusion chromatography (IEC) was applied on an 883 Ion Chromatograph (IC; Metrohm, Switzerland), using a Transgenomic IC Sep ICE-ION-300 column (30 cm length, 7.8 mm diameter and 7 μ m particles) and a MetroSep RP2 Guard. The mobile phase consists of 1.5 mM aqueous sulphuric acid. A column flow rate of 0.4 ml*min⁻¹ was used. The temperature of the column was 65 °C. The organic acids were detected using suppressed conductivity detection. Samples were centrifuged (21,000 g, 10 min) and the clear supernatant was filtered through a 0.45 μ m PFTE filter and diluted with mobile phase (for lumen 1:5, for dialysate 1:2). Ten microliters were loaded on the column by an autosampler 730 (Metrohm). Molecules were eluted according to their pKa.

Constituent monosaccharide composition. The constituent monosaccharide content and composition were determined using a prehydrolysis step with 72 % (w/w%) sulfuric acid at 30 °C for 1 h followed by hydrolysis with 1 M sulfuric acid at 100 °C for 3 h. The monosaccharides formed upon hydrolysis were derivatized to alditol acetates and analyzed by gas chromatography using inositol as internal standard ²⁶. The colorimetric *m*-hydroxydiphenyl assay was used to determine the total uronic acid content ²⁷.

Molecular weight distribution. Fermentation digests (corresponding to 2 mL lumen samples) or dry raw materials, which were dissolved in ultrapure water, were centrifuged (10 min, 18,000 g, 24 °C) to obtain the soluble fraction, which was analyzed for molecular weight distribution using high-performance size exclusion chromatograph (HPSEC) on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA). Three SK-Gel columns in series (4000-3000-2500 Super AW; 150 X 6 mm) were used for the analysis. All columns were from Tosoh Bioscience (Tokyo, Japan). Pullulan molecular-mass standards (Polymer Laboratories, Palo Alto, CA, USA) were used for calibration ²⁸.

Oligosaccharide Profiling. High-performance anion exchange chromatograph (HPAEC) was performed on an ICS5000 system (Dionex), equipped with a Dionex CarboPac PA-1 column (2 X 250 mm) in combination with a CarboPac PA-1 guard column (2 X 250). The flow rate was 0.3 mL/min with an eluent profile starting with 0.02 M NaOH until 13 min, then increasing to 0.1 M NaOH until 15 min, followed by a linear gradient of 0-500 mM NaOAc in 0.1M NaOH until 45 min, followed by a gradient to 1 M NaOAc in 0.1 M NaOH in 1 min and 7 min at 1M NaOAc in 0.1 M NaOH. After this, the column was equilibrated with 0.1 M NaOH for 3 min and 0.02 M NaOH for 20 min. An ICS5000ED (Dionex) Pulsed Amperometric Detector and Chromeleon software version 7 were used. Oligomers of cellulose (DP 2-DP 6) were used as standards to identify cellulose oligomers in the elution profile.

Statistics

Kruskal-Wallis Rank Sum Test (one-way ANOVA on ranks) was applied to compare alpha diversities (Shannon index and Faith's PD) among different RSM treatments, and Wilcoxon Rank Sum Test was used for pairwise comparison in R version 3.5.3 (<https://www.r-project.org/>). Bonferroni adjustments were used to correct P-values for multiple comparisons. Permutational multivariate analysis of variance (PERMANOVA) was performed to test the significance of beta diversity (weighted and unweighted UniFrac) between non-processed and processed RSM in QIIME2. The results were visualized in R.

The ASV table (feature table of QIIME2) was normalized and filtered in R and statistical analysis was performed in STAMP²⁹. The table was normalized via division by the sum of sequences in a given sample and multiplied by the minimum sum across all samples. Relative abundances were filtered as following: values below a relative abundance threshold of 0.01% were not taken into account; taxa with a median relative abundance <1 % in all groups were not considered for statistical analysis. White's non-parametric t-test was applied to compare between CON group and treatments. *P*-values were corrected using the Benjamini-Hochberg method. A *q*-value (corrected *P*-value) < 0.05 was considered significant.

Pearson correlations between continuous meta-variables and taxonomic variables were calculated and visualized in R (R version 3.5.3). Parameters were set as following: Missing values for meta-variables were handled as NO imputation (replacing missing data with substituted); zeros were kept for the calculation of correlation; a minimum number of 0.1 % was considered for calculation; a minimum of 4 paired observations were required for calculation of correlations.

T-tests were conducted to compare SCFA production between CON and the treated RSM substrates in the built-in R package (R version 3.5.3).

Results

Description and characteristics of untreated and processed RSM

Table 1 shows the constituent monosaccharide composition of (processed) RSM. The carbohydrate content of CON is 62% w/w. Dominant sugars are glucose (Glc, 31 mol %), uronic acid (UA, 19 mol %), arabinose (Ara, 5 mol %), and galactose (Gal, 11 mol %). CON contained 56 % pectin (defined as: rhamnose + arabinose + galactose + uronic acid) and 44 % (hemi)cellulose (xylose + mannose + fucose + glucose). The values of pectin and (hemi)cellulose for ALK_A, ALK_B, PECT1_A, PECT1_B, PECT2_A, PECT2_B, CELL_A, and CELL_B were 55 % and 45%, 52 % and 48%, 54 % and 46 %, 50 % and 50%, 56 % and 44 %, 51 % and 49 %, 57 % and 43 %, and 60 % and 40 %, respectively. Relative pectin contents decreased in ALK, PECT1, and PECT2 treatment, while they increased in CELL treatment, compared to CON. ALK, PECT1, and PECT2 treatment increased (hemi)cellulose values, compared to CON. Predigesting before or after processing RSM had little effect on the monosaccharide constitution.

Table 1. Constituent monosaccharide composition of processed RSM

	(mol%)								(w/w%)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total
CON	1	1	25	10	2	11	31	19	62
ALK_A	1	1	23	9	3	11	35	17	58
ALK_B	1	1	26	9	2	8	32	21	52
PECT1_A	1	1	26	9	2	14	37	9	61
PECT1_B	1	1	22	8	2	10	34	21	53
PECT2_A	1	1	16	8	3	11	37	22	63
PECT2_B	1	1	23	9	3	11	31	21	57
CELL_A	1	1	24	9	2	11	28	23	63
CELL_B	1	1	24	9	3	10	30	23	56

Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, Uronic acid. _A, RSM was treated after predigesting, _B, RSM was treated before predigesting.

Considerable changes occurred in the microbiota fed with ALK- and CELL-processed RSM after a shot of 5 grams of test products.

Changes in the gut microbiota in response to a shot of 5 grams of the different treated RSM substrates were determined. When data from all of the time points were pooled, Shannon index of ALK and CELL were significant lower than CON, while there were no significant differences for PECT1 and PECT2 compared to CON (Figure 2). Within the same group, different time points did not appear significantly different in term of Shannon index (data not shown). Phylogeny based UniFrac distance matrix measurements were then used to compare the β -diversity of the microbial communities between microbiota fed with non-processed and processed RSM. Unweighted UniFrac, which is clustering data based on presence or absence of ASV, clustered the non-processed and processed RSMs samples separately ($P < 0.001$). There was no clear separation between CON and PECT1, and between CON and PECT2 in terms of weighted UniFrac metrics ($P > 0.05$), which also considers the relative abundance of the ASV, while CON was significantly different with ALK and CELL ($P < 0.001$) (Figure 3, and S1). Both weighted and unweighted UniFrac revealed that bacterial community structure of CON were more similar to PECT1 and PECT2 than those of ALK and CELL, while microbial community compositions of PECT1 and PECT2 were similar to

each other ($P_{\text{weighted UniFrac}} = 0.131$, $P_{\text{unweighted UniFrac}} = 0.078$) (Supplemental file 2: Figure S1).

Figure 2. Community diversity represented by Shannon index at ASV level for samples from each treatment. Shannon index was calculated based on the average of ten iterations at equal sampling-depth 7139 for each sample. Each bar represents the samples from the microbiota fed with non-processed RSM (CON) and RSM processed by Accellerase 1000 (CELL), Pectinex Ultra SP (PECT1), Multifect Pectinase (PECT2), or 6M NaOH (ALK).

Figure 3. Principle coordinate analysis (PCoA) plot generated based on the calculated distances in the unweighted matrix. Samples were grouped by shape and colour in terms of treatment and time point, respectively: non-processed RSM (CON), square; RSM processed by 6 M NaOH (ALK), sphere; RSM processed by Pectinex Ultra SP (PECT1), plus; RSM processed by Multifect Pectinase (PECT2), square with cross inside; RSM processed by Accellerase 1000 (CELL), triangle; a red-green-purple scale was used to indicate the fermentation time (red and purple depict start and end of the fermentation period).

Alpha-diversity (Shannon index) and β -diversity (weighted and unweighted UniFrac) showed that there were no significant differences between microbiota fed with processed RSM before or after dialysis (data not shown). This indicated that predigesting before or after processing RSM by ALK or enzymes, did not result in different microbiome compositions. Furthermore, predigesting before or after processing RSM also had little effect on their monosaccharide constitution as described above. Therefore, they were treated as duplicates in the (following) microbial relative abundance analysis.

We next compared microbial relative abundance of CON with those of the microbiotas fed with the different processed RSMs to identify significantly different bacterial taxa. Data from all time points were grouped. No significant differences were detected at the phylum level when comparing CON to the other groups (ALK, PECT1, PECT2 and CELL) (data not shown). At the genus level, when compared to CON, ALK treatment resulted in significantly

increased relative abundance of *Olsenella* ($P = 0.017$), *Runimicoccus gauvreauii* group ($P = 0.019$), *Eubacterium nodatum* group ($P < 0.001$), *Megasphaera* ($P < 0.001$), *Bifidobacterium* ($P < 0.001$), *Acidaminococcus* ($P < 0.001$), and *Acetitomaculum* ($P < 0.001$), which were from the phyla Actinobacteria and Firmicutes. In addition ALK treatment significantly decreased the relative abundance of *Ruminococcaceae* UCG-002 ($P < 0.001$), *Christensenellaceae* R-7 group ($P < 0.001$), *Enterobacteriaceae* unknown group ($P < 0.001$), *p-2534-18B5* gut group from the order Bacteroidales ($P < 0.001$), *Citrobacter* ($P < 0.001$), *Prevotella* 9 ($P = 0.004$), *Rikenellaceae* RC9 gut group ($P = 0.006$), *Desulfovibrio* ($P = 0.007$), *Prevotellaceae* NK3B31 group ($P = 0.030$), and *Lachnoclostridium* ($P = 0.031$), which were from the phyla Bacteroidetes, Firmicutes and Proteobacteria (Figure 4 and Supplemental file 2: Figure S2).

Figure 4. Significantly different microbial genera in relative abundance in the different treatments compared to CON. White's non-parametric T-test was applied to compare between CON group and treatments. P -values were corrected using the Benjamini-Hochberg method (q -values). The shown mean relative abundance percentages of the taxa were calculated using all samples taken over time within each treatment.

PECT2 treatment significantly decreased the relative frequencies of *Enterobacteriaceae* unknown group ($P < 0.001$) and *Christensenellaceae* R-7 group ($P < 0.001$) when compared with CON group (Figure 4). There was no significant difference at genus level between CON group and PECT1 treatment group.

Relative abundance of *Olsenella* ($P < 0.001$), *Eubacterium nodatum* group ($P < 0.001$), *Acidaminococcus* ($P < 0.001$), *Lachnospiraceae* NK3A20 group ($P < 0.001$), *Bifidobacterium* ($P < 0.001$), *Acetitomaculum* ($P = 0.014$), and *Syntrophococcus* ($P = 0.016$), from the phyla Actinobacteria and Firmicutes, significantly increased in microbiota fed CELL-processed RSM. Moreover, CELL treatment decreased the relative abundance of *Ruminococcaceae* UCG-002, *Christensenellaceae* R-7 group, *p-2534-18B5* gut group from the order Bacteroidales, *Rikenellaceae* RC9 gut group, and *Succiniclasticum*, which were from Firmicutes and Bacteroidetes (Figure 4 and S2).

ALK and CELL significantly increased the microbial functional abundances related to fibre degradation and SCFA production compared to CON.

PICRUSt2 was performed to the 16S rRNA gene data to predict metagenomic functional profiles. Compared with CON, 111 features were significantly different in ALK, 108 features

in CELL, 2 features in PECT1 and 1 feature in PECT2 (Supplemental file 2: Figure S3). As the current study focused on fibre degradation, only carbohydrate metabolism related microbial functional features are summarized. The relative abundances of carbohydrate digestion and absorption ($P = 0.047$), galactose metabolism ($P = 0.008$), glycolysis ($P = 0.001$), pentose phosphate pathway ($P = 0.004$), propanoate metabolism ($P < 0.001$), and pyruvate metabolism ($P < 0.001$) were predicted to be significantly higher upon ALK feeding, while glycan biosynthesis and metabolism ($P = 0.005$), and lipopolysaccharide biosynthesis ($P = 0.005$) were significant higher in CON (Figure 5A). After the microbiota was fed with CELL, microbial functional abundance of carbohydrate digestion and absorption ($P = 0.019$), energy metabolism ($P = 0.018$), fructose mannose metabolism ($P = 0.020$), galactose metabolism ($P = 0.010$), glycerolipid metabolism ($P < 0.001$), glycolysis ($P < 0.001$), pentose phosphate pathway ($P = 0.004$) and pyruvate metabolism ($P = 0.001$) significantly increased (Figure 5B). There were no significant changes in carbohydrate metabolism related microbial abundance upon feeding PECT1 or PECT2 when compared to CON.

Figure 5. Significantly different metagenomic functions in relative abundance in the treatments ALK (A) and CELL (B) compared to CON. Difference of short-chain fatty acid production (C) during fermentation of ALK, PECT1, PECT2, and CELL compared to CON.

Cumulative short-chain fatty acid production by the microbiota fed with CON was compared with those by the microbiotas fed with ALK, PECT1, PECT2 or CELL. The amount of propionic acid ($P_{\text{ALK}} = 0.010$, $P_{\text{CELL}} = 0.006$) and the total SCFA ($P_{\text{ALK}} = 0.008$, $P_{\text{CELL}} = 0.015$) in ALK and CELL were significant higher than those in CON, while there were no significant differences in SCFA production in PECT1 and PECT2 compared with CON.

(Figure 5C).

Random Forest revealed a RSM degradation pattern in a porcine gut microbiota.

The supervised machine learning technique Random Forest was applied to predict fermentation time. Every possible time interval was used {e.g. (48, 49] (49, 72], (48, 50](50, 72], (48, 52] (52, 72], (48, 54] (54, 72], and (48, 56] (56, 72]}, but only the time interval, (48, 52] (52, 72], performed well at the prediction task (Supplemental file 3: Table S1). The Random Forest proximity plot shows that samples from time point 48.5 to 52 were clustered (Figure 6A). This indicated that microbiota composition considerably changed only after incubation for 4 hours after a shot of 5 grams of treated RSM, which may have occurred because the nutrient composition in the lumen significantly changed. To further understand the Random Forest model, the ASV with the most influence in the Random Forest prediction was identified, which turned out to be a genus in the family Veillonellaceae: *Megasphaera* (Figure 6B). *Megasphaera* was also significantly higher in ALK when tested with the Kruskal-Wallis non-parametric test (Figure 4).

Figure 6. Random Forest proximity plots of time points (A) and treatments (C), and the ASV with the most influence in the random forest prediction (B for time points, D for treatments). To generate this representation, a distance was calculated between samples based on how frequently samples occur in the same tree partition in the random forest's bootstrapping

procedure. If a pair of samples frequently occurred in the same partition, the pair was assigned a low distance. The resulting distances are then input to the PCA.

Interestingly, the effect on the microbiota composition of the different processing methods on RSM was also predicted by Random Forest. Table S2 (Supplemental file 2) shows that Random Forest performed well at this prediction task, and the Random Forest proximity plot demonstrates that microbiota fed with CON had less difference with PECT1 and PECT2, while there was a larger distance with ALK and CELL (Figure 6C). The microorganisms with the most influence in classifying the different processing methods was in family Ruminococcaceae: *Ruminococcaceae* UCG-002 (Figure 6D).

HPSEC elution profiles showed almost complete degradation of soluble high molecular weight polysaccharides at time point 52 h.

Molecular weight distributions of soluble fibres from RSM with different processing methods are shown in Figure 7. For ALK, PECT1, PECT2 and CELL fermentation, an increase in the amount of soluble materials corresponding to high molecular weight (M_w) was seen 2, 1, 1, and 1 h after the shot (at time point 50 or 49 h), respectively. While for CON, the increase in soluble materials with high M_w , was seen already after 0.5 h of incubation (at time point 48.5 h). Although high M_w material was observed before the shot of 5 grams RSM (at time point 48), indicating that the starvation period did not lead to complete fermentation of the fibres, as observed before³⁰, they were degraded rapidly after 0.5 h (at time point 48.5 h), since the peaks of 48.5 h were lower than those of 48 h, which can be seen in the HPSEC profiles of ALK, PECT1, PECT2 and CELL. From 4 h onward (at time point 52 h), an almost complete disappearance in the high M_w fraction occurred, indicating degradation and/or utilization of all high M_w polysaccharides.

Figure 7. HPSEC elution patterns of soluble fraction of fermentation digests during fermentation of CON (A), ALK (B), PECT1 (C), PECT2 (D), and CELL (E). Pullulan was used as a calibrant.

Compared to the highest peak of the elution profile of CON (48.5 h), the highest peaks of the elution profile of ALK and CELL (50 h and 49 h, respectively) showed a higher amount of high M_w material, while those of the elution profile of PECT1 and PECT2 were

approximately equal to CON (Figure 7). This indicated that more materials were solubilized under the conditions when ALK and CELL were fed, than with the other substrates.

HPAEC elution profiles indicated large amount of soluble oligo-celluloses formed during ALK and CELL fermentation.

To determine oligomers formed and utilized during fermentation of processed versus non-processed RSM, soluble fractions from fermentation digests were analyzed using HPAEC (Figure 8). For ALK and CELL, a few peaks can be identified as oligomers of cellulose on the basis of standards of cellulose oligomers as indicated in Figure 8 (cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose). However, most of the peaks in ALK and CELL fermentation samples were not identifiable on the basis of the cellulodextrin standard. These oligomers are only present upon ALK and CELL feeding, and were not seen with PECT1, PECT2 and CON. The oligomers were increased to highest amounts 0.5 h after addition of the 5 g shot (time point 48.5 h), and decreased to be almost completely fermented after 8 h (time point 56 h).

Figure 8, HPAEC elution profile of fermentation digests during fermentation of CON (A), ALK (B), PECT1 (C), PECT2 (D), and CELL (E). C2: D-(+) cellobiose; C3: 1,4- β -D-cellotriose; C4: 1,4- β -D-cellotetraose; C5: 1,4- β -D-cellopentaose; C6: 1,4- β -D-cellohexaose.

No pectin oligomers were observed in both enzymatic and chemical treatment groups.

Constituent monosaccharide composition of fermentation samples showed that less residual carbohydrates remained in ALK and CELL compared to CON.

Direct utilization of polysaccharides during RSM fermentation can be revealed from the decrease in the carbohydrate content. Figure 9 shows the utilization of the main monosaccharides in RSM, which are arabinose, galactose, glucose, and uronic acid. The amount of main monosaccharides was lower after 24 h fermentation (at time point 72) for ALK and CELL compared to CON. The utilization of the main monosaccharides plateaued after time point 52, except for glucose, which was continuously utilized until time point 72. The utilization of arabinose and galactose were faster in ALK and PECT1, compared to the other treatments.

Figure 9. Utilization of arabinose (Ara), galactose (Gal), glucose (Glc), and uronic acid (UA) present in CON (A), ALK (B), PECT1 (C), PECT2 (D), and CELL (E) during *in vitro* fermentation. Values presented are means of duplicates measurements.

Correlation between microbiota abundance and SCFA production and monosaccharide composition.

Correlations between the relative abundances at genus level and SCFA production and monosaccharide composition (mg/mL sugar left) at each time point were analyzed (Figure 10). *Bifidobacterium*, [*Eubacterium*] *nodatum* group, and *Acidaminococcus* had significant negative correlations with acetic and butyric acid, while *Prevotella* 7 and *Megasphaera* had significant positive correlation with acetic, propionic and butyric acid. Butyric acid significantly positively correlated with *Prevotellaceae* NK3B31 group and *Desulfovibrio*.

Figure 10. Correlation between core bacterial genera and SCFA production and molar percentage of monosaccharides. Statistical significance was determined for all pairwise comparisons using Spearman's method. The relative abundances of ASVs were significantly negatively correlated with monosaccharides (mg/mL sugar left in the lumen), which indicated the more bacterial abundance, the more utilization of these monosaccharides. For instance, *Prevotella 7* was significantly negatively correlated with Rha, which means that more Rha was utilized when the relative abundance of *Prevotella 7* increased. Side chain versus backbone pectin = (Ara + Gal) : (galA+Rha), Fate of side chains = Ara : Gal, (Hemi)cellulose versus pectin = (Xyl + Glc + Man) : (galA + Gal + Ara + Rha). Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose, Man, mannose, Gal, galactose, Glc, glucose; galA, assumed to be equal to uronic acid; Total, the total sugar left in the lumen * $P < 0.05$; ** $P < 0.01$. Size of the circles means correlation values, which bigger sizes meaning higher correlation values. Blue circles represent positive correlations, whereas red circles represent negative correlations.

Rha, Ara, Xyl, Man, Glu, and total monosaccharides (Total) had significant negative correlations with *Prevotella 7* and *Megasphaera*, and Gal and UA also had significant negative correlations with *Megasphaera*. *Olsenella* was significantly negatively correlated with 'Side chain versus backbone pectin' [= (Ara + Gal) : (galA+Rha)], while [*Ruminococcus*] *gavreaii* group and *Desulfovibrio* had significant positive correlation with this. *Bifidobacterium* was significantly positively correlated with 'Fate of side chains' [Ara : Gal], whereas *Succinibacterium* and *Megasphaera* had significant negative correlation with this. *Bacteroidales* S24-7 group and unknown genera from family Prevotellaceae and Enterobacteriaceae had significant negative correlation with '(Hemi)cellulose versus pectin' [(Xyl + Glc + Man) : (galA + Gal + Ara + Rha)], while *Prevotella 7* and Rikenellaceae RC9 gut group were significantly positively correlated with this.

MNBT validated that more fibres were degraded in case of ALK and CELL treatment, compared to PECT1 and PECT2.

Utilization of cell wall polysaccharides upon RSM fermentation assessed using the ileal cannulated growing pigs can be revealed from the decrease in the total carbohydrate content as well as from the constituent monosaccharides of the material in the collected bags after transit through the pigs (Table 2). After the nine substrates (in the nylon bags) passed through the ileal cannulated growing pigs, there were 31 % (CON), 11 and 22 % ALK_A and ALK_B, 30 and 31 % PECT1_A and _B, 30 and 30 % PECT2_A and _B, and 19 and 22 % CELL_A and _B carbohydrates left in the residues, respectively (calculated from the sugar composition and the recovery of DM). The constituent monosaccharides of non-processed and processed RSM were similar to each other (Table 1), while mol percentages of Ara, Glc and Gal were shifted in ALK and CELL after fermentation *in sacco* (Table 2). The percentages of Ara and Gal were lower in ALK and CELL after fermentation, where the mol percentages of Ara decreased from 23 % to 9 % for ALK_A, 26 % to 5 % for ALK_B, 24 % to 5 % for CELL_A and from 24 % to 10 % for CELL_B (Table 1 and Table 2). Molar percentages of Gal were diminished from 11 % to 6 % in ALK_A, from 8 % to 4 % in ALK_B, 11 % to 6 % in CELL_A, and from 10 % to 7 % in CELL_B. Glc was increased from 35 % to 37 % in ALK_A, from 32 % to 68 % in ALK_B, from 28 % to 50 % in CELL_A, and from 30 % to 37 % in CELL_B. On top of this change in mol percentages, the recovery of DM was also reduced by at least 69%, as described above, indicating e.g. that for ALK_A (11% DM recovery) Ara decreased by approximately 25-fold (Supplemental file 2: Table S3).

Table 2. Constituent monosaccharide composition of residues obtained from *in sacco* fermentation of CON, ALK, PECT1, PECT2 and CELL in ileal cannulated pigs.

	(mol%)								w/w % Total	(w/w %) Recovery of DM
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA		
CON	2	1	17	9	2	10	40	20	87	36
ALK_A	2	1	9	13	2	6	37	30	82	13
ALK_B	1	1	5	2	1	4	68	18	80	27
PECT1_A	2	1	19	9	2	11	46	10	87	35
PECT1_B	2	1	18	11	2	10	39	17	89	35
PECT2_A	2	1	10	13	2	5	39	28	86	35
PECT2_B	3	1	13	12	2	6	32	30	86	35
CELL_A	3	1	5	10	3	6	50	23	83	23
CELL_B	3	1	10	10	2	7	37	30	81	27

Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, Uronic acid. _A, RSM was treated after predigesting, _B, RSM was treated before predigesting.

PECT1, PECT2, and CON had less composition shift in these monosaccharides compared to ALK and CELL. Molar percentages of Ara decreased from 25 % to 17% for CON, from 26 to 18% for both PECT1_A and PECT1_B, from 16 % to 10 % for PECT2_A, and from 23 % to 13% for PECT2_B. The changes of Gal were within 2 % for PECT1, PECT2, and CON. Molar percentage of Glc in PECT1, PECT2, and CON increased by 7%, 6%, and 2 % on average, respectively. This does not mean that the microbiota did not ferment these substrates though. Since the recovery of total carbohydrate for these substrates in sacco is 30% (see above), it means that 70% has been fermented. Despite this, the remaining carbohydrate has a similar monosaccharide composition as the original substrate that was inserted in the ileum.

Discussion

Our *in vitro* studies on the swine microbiota demonstrated that feeding the microbiota with RSM processed with two kinds of pectinases (PECT1 and PECT2), a cellulase (CELL), or ALK, induced differences in the composition and functionality of the gut microbiota compared to CON. Our findings revealed that ALK and CELL significantly increased the microbial functional abundances related to fibre degradation and also increased SCFA production compared to CON, which did not occur with PECT1 and PECT2. This is in line with the greater reduction of monosaccharide amounts in the nylon bag experiments.

Alpha diversities of ALK and CELL were lower compared to CON, while there were no significant difference among PECT1, PECT2 and CON (Figure 2), which may be due to the selection of particular genera in Actinobacteria and Firmicutes³¹. The abundances of many microbes after feeding with CELL and ALK were significantly higher than for CON, while abundances were not shifted with PECT1 and PECT2 (Figure 4). It has been shown that exogenous carbohydrases from *Trichoderma longibrachiatum* help in degradation of specific bonds of cell walls either before or after ingestion of the enzyme preparation, and in turn this caused an increase in the numbers and/or activities of bacteria that utilize the polysaccharides in the GIT^{13, 32}. Supplementation of carbohydrases has been shown to modulate gut microbiota, in various animal models, or *in vitro*¹³⁻¹⁵. Another possible mode of action of carbohydrases that has been shown in the rumen is that the enzyme preparation alters the fibre structures of substrate and stimulates the attachment of rumen microbiota to feed particles, resulting in improved the fibre degradation¹⁴. Giraldo et. al (2008) also reported that supplementing carbohydrases directly into the rumen increased the overall fibrolytic activity and stimulated the growth of cellulolytic bacteria¹⁵. In the current study, CELL treatment (prior to ingestion) significantly increased the numbers of *Olsenella*, [*Eubacterium*] *nodatum* group, *Acidaminococcus*, *Lachnospiraceae* NK3A20 group, *Bifidobacterium*, *Acetitomaculum*, and *Syntrophococcus* (Figure 4). These genera may prefer to utilize cellulose- and/or hemicellulose-fragments generated by the action of CELL. However, in the literature only *Eubacterium* has been reported as cellulolytic³³. CELL contains multiple glycolytic activities, including exo-1,4- β -glucanase (cellobiohydrolase), endo-1,4- β -glucanase, hemicellulase and β -glucosidase. CELL may have broken down some bonds in cellulose thereby enhancing (hemi)cellulose utilization by the gut bacteria and at the same time also expose other polysaccharides (e.g. pectins) to other bacteria. ALK increased *Megasphaera*, and *Ruminococcus gnavus* group. Reports show that *Ruminococcus* are the most commonly cellulolytic organisms³³⁻³⁶. *Megasphaera* is reported to contain glycosyl hydrolase (GH) family 53, which is involved in plant cell wall degradation³⁷. These reports indicate that ALK can disrupt the cell wall architecture, by solubilization of polysaccharides by breaking hydrogen bonds and hydrolyzing ester linkages thereby removing esters present as decoration on polysaccharides, making them more accessible for further enzyme degradation and utilization by the microbiota. Interestingly, both PECT1 and PECT2 showed no significant difference in Shannon index compared to CON and a very similar microbiota composition, which seemed to indicate that neither pectinase really changed the cell wall structure, or the changes did not meet the swine gut microbiotas' hydrolytic capacities, and as a result no bacteria were selectively stimulated. We reported before that a cocktail of PECT1 and PECT2 improved degradability of non-glucose polysaccharides of RSM in broilers³⁸⁻³⁹. Nevertheless, it seems that cell wall break down by PECT1 and PECT2 does not lead to advantages for members of the swine gut microbiota.

The shifts in the bacterial community structures were converted into predicted functional metagenomic profiles (Figure 3, 5 and 6C). PECT1 and PECT2 had little effect on the predicted microbial function, as expected, since there were few changes in the microbial structure. In contrast, CELL and ALK had greater microbial composition shifts, subsequently resulting in more changes in microbial function. In the current study, we were interested in fibre utilization, thus, the significantly different microbial functions of carbohydrate metabolism were summarized (Figure 5). The abundances of the carbohydrate related microbial functional metabolism pathways, pyruvate metabolism, propanoate metabolism, pentose phosphate pathway, galactose metabolism, energy metabolism, fructose and mannose metabolism, and carbohydrate digestion and absorption (Figure 5), were higher in CELL and/or ALK compared to CON, while the abundances of lipopolysaccharide biosynthesis and glycan biosynthesis and metabolism were higher in CON compared to ALK. This was corroborated by the SCFA production, which showed that the total SCFA and propionic acid production were significant higher in CELL and ALK compared to CON (Figure 5). Giraldo et al.¹⁴ reported that supplementation of endoglucanase and xylanase increased propionate production by 28 %, and total SCFA production by 11%. Thus, the hypothesis can be entertained that the complementary action between the stimulated microbes and prior incubation with the exogenous enzymes leads to the increase in hydrolytic capacity⁴⁰. However, another study⁴¹ demonstrated that the concentration of cellulolytic bacteria was not the limiting factor in the digestion of cellulose, and reported that factors associated with the forage and/or the rate of cellulose hydrolysis by cellulase may have a greater influence on the amount of cellulose digested in the rumen. According to the current study, a prerequisite for the complementary action in breaking down recalcitrant fibres might be that the cell wall structure of the substrate is processed appropriately by the feed enzyme, thereby making it more amenable to subsequent degradation by the gut microbial enzymes. HPAEC showed that oligomers of cellulose were detected upon feeding CELL and ALK, which were not seen with PECT1, PECT2 and CON (Figure 8). Our *in sacco* study also showed that more carbohydrate was utilized upon feeding CELL and ALK, based on dry matter recovery. Moreover, constituent monosaccharide compositions shifted with CELL and ALK. This did not occur with PECT1, PECT2 and CON (Table 2). This can be explained by the fact that CELL and ALK broke down cellulose microfibrils, and stimulated fibrolytic bacteria, which expressed more related enzymes. At the end, more SCFA were produced. This might also be why SCFA production was not increase with PECT1 or PECT2, since no oligomers of pectins were detected in HPAEC (Figure 8). However, the mechanism of exogenous enzymes enhancing degradation of plant cell walls is complex with many interrelated factors, and requires further studies⁴². Moreover, degradation of fibres requires a plethora of microbial enzymes as indicated by the numerous PUL-loci needed by *Bacteroides thetaiotaomicron* to breakdown pectin⁴². So, this future research needs to elucidate these interrelated factors, since a better understanding of the mode of the action will allow the development of feed enzymes designed specifically to improve feed digestion by swine.

Random Forest analysis found that the microbiota structures were significantly changed after 4 hours fermentation after a shot of processed or non-processed RSM (Figure 6A). This can be explained by comparing this with the results of HPSEC and HPAEC, which showed that almost all the high molecular weight polysaccharides fibres were utilized after 4 hours fermentation (time point 52 h), and converted into low molecular weight sugars which are

substrate for the microbiota. The changes of nutrient composition led to the shifts of microbiota structure. *Megasphaera* was identified by Random Forest as the most important genus during the classification. Figure 6B shows that a large number of samples contained *Megasphaera* during the fermentation period. This indicates that *Megasphaera* might be a microbe, which can utilize the non-processed and processed RSM well. Genome-wide analysis of *Megasphaera* sp. showed that the genomes had genes coding GH25, GH32, GH43, GH53, GH73 and GH77, which indicate its ability to degrade complex carbohydrates^{37, 43}. *Megasphaera* is also known to produce all of the SCFA including valerate^{37, 43-44}. In the current study, the correlation between SCFA production and relative abundance of genera also showed that *Megasphaera* had significantly positive correlation with acetic acid and propionic acid (Figure 10). *Prevotella* 7, and *Desulfovibrio* were also significantly positively correlated with SCFA production (Figure 10), while *Megasphaera* and *Prevotella* 7 had considerably negative correlations with the 'Fate of side chains' (Ara : Gal), and/or had positive correlations with '(Hemi)cellulose versus pectin' [(Xyl + Glc + Man) : (galA + Gal + Ara + Rha)]. This indicated that these genera had the ability to use the side chain of pectins in the RSM cell wall, and produced SCFA, but only after degradation of the cellulose network, as this was not seen for substrates treated with PECT1 and PECT2. *Prevotella* 7 and *Megasphaera* also had significant negative correlation with most of the monosaccharides, which indicated they can use the monosaccharides well. Research showed that *Desulfovibrio* significantly increased after being fed RG-I-enriched pectin, and SCFA production was also found increased⁴⁵. *Prevotella* is well known as an important pectinolytic bacterium⁴⁶⁻⁴⁸. So, removal of cellulose by CELL seems to increase accessibility of microbial enzymes to pectin. However, in literature, reasoning the other way around is more frequently done: pectinases to remove pectins from the pores from the cell wall enhancing the activity of cellulases³⁰.

In conclusion, CELL and ALK feeding considerably changed the swine microbiota structure and predicted functional profile compared to CON, which did not occur with PECT1 and PECT2. The hypothesis is entertained that this results from the different cell wall architecture of RSM once processed by this carbohydrase or alkaline. The increase in relative abundance of pathways involved carbohydrate fermentation in CELL or ALK can be considered as a positive effect of these treatments in fibre utilization and SCFA production. Moreover, it was validated in pigs that CELL and ALK feeding improved the overall degradation of RSM by the mobile nylon bag technique. With all the data collectively considered, we speculate that carbohydrase enzyme, i.e. CELL, improve fibre degradation of RSM during the fermentation by changing the microbial community structure and enzymatic activity and subsequently shifting the microbiota metagenomic functional profile.

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Supplemental figures:

Figure S1. Principle coordinate analysis (PCoA) plot generated based on the calculated distances in the weighted matrix. Samples were grouped by shape and colour in terms of treatment and time point, respectively: non-processed RSM (CON), square; RSM processed by 6 M NaOH (ALK), sphere; RSM processed by Pectinex Ultra SP (PECT1), plus; RSM processed by Multifect Pectinase (PECT2), square with cross inside; RSM processed by Accellerase 1000 (CELL), triangle; a red-green-purple scale was used to indicate the fermentation time (red and purple depict start and end of the fermentation period).

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Figure S2. Phylogenetic tree of the core bacteria fed with non-modified and modified RSM. The table underlying the tree was normalized via division by the sum of sequences in a given sample and multiplied by the minimum sum across all samples. Relative abundances were filtered as following: values below a relative abundance threshold of 0.01% were not taken into account; taxa with a median relative abundance <1 % in all groups were not considered for statistical analysis; purple squares represents the relative abundance of the ASVs higher than 0.5 %, while the yellow triangle stands for those lower than 0.5 %. The outer ring heatmap stands for the microbial relative abundance. Light green indicates lower abundance, while dark green represents higher abundance.

Chapter 5

Figure S3: Significantly different metagenomic function in relative abundance in different treatment compared to CON

Figure S3: Significantly different metagenomic function in relative abundance in different treatment compared to CON

Chapter 6

High-throughput screening of structures of plant cell wall polysaccharides using a novel method

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Abstract

The aim of the study was to develop a methodology, which enables to detect and determine plant cell wall structures in plant materials. The technique (glycome profiling of plant cell wall polysaccharides, or Glycoprofiling) is developed with the use of carbohydrate binding modules (CBMs) with unique specificities for plant cell wall polysaccharides, linked to a green fluorescent protein (GFP) as reporter. Different plasmid constructs with 35 different CBMs were made. These were overexpressed in *E.coli* and purified with the use of a HIS-tag. The fusion proteins were then tested against a panel of 40 carbohydrates. When used on plant material, the profiling results would provide a global overview of the cell wall polysaccharide composition and structure. The toolbox can be used to extend the monoclonal antibody repertoire that we used in Chapter 3, and can easily be extended with additional CBM-fusion proteins. Moreover, fusion proteins with different colors (e.g. m-Cherry (red), YFP (yellow) or BFP (blue)) can be made by simple exchange with GFP. This toolbox may become essential in the study of the dynamics of breakdown of dietary fibres by the (pig) gut microbiota.

Introduction

Plant cell wall polysaccharides are composed of (hemi)celluloses and pectins. Cellulose is crystalline and strong, thus it is an important structural component of the primary cell wall in green plants. Hemicelluloses bind with pectin and cellulose to form a network of cross-linked fibres. Pectins are linked to each other and cross-linked between pectin and hemicellulose, and between pectin and cellulose (1). Pectins consist of homogalacturonan (HG), rhamnogalacturonan I and II (RG-I and RG-II), xylogalacturonan, arabinogalactan type I and type II (AG-I and AG-II) and arabinan. Hemicelluloses are composed of xylan, xyloglucan, mannan, and β -glucan. The plant cell wall is a potential energy source as animal feed for livestock. However, plant cell wall polysaccharides cannot be utilized by endogenous enzymes in animal, and also can only be partly degraded by gut microbiota. Therefore, in order to develop new methods to improve the degradability of plant cell wall polysaccharides, a better understanding of the cell wall polysaccharide composition and structure and their interactions are essential.

One of the promising high-throughput way to screen the polysaccharide structure in plant cell wall is by the use of carbohydrate binding modules (CBMs) and/or monoclonal antibodies (mAbs) (2). CBMs are the non-catalytic part of cell-wall-degrading enzymes, and they are attached to the glycoside hydrolase (GH) catalytic modules. Usually, CBMs have to recognize and bind to the specific polysaccharides first, before the GHs cleave the polysaccharides (3). CBMs are grouped into amino acid sequence-based families in the Carbohydrate-Active EnZymes (CAZy) database (<http://www.cazy.org/>) (4), and currently, there are 87 CBM families in the database. CBMs have been applied to recognize xylans (5), cellulose (6, 7), and mannans (7) directly in plant materials.

Another important tool for study of plant cell wall structure is using immunohistochemical techniques (e.g. mAbs) (8), which is developed from neoglycoprotein immunization procedures or from retrospective characterization after immunization with complex cell wall-derived materials (9). Repertoires of cell wall-directed monoclonal antibodies are being assembled. For example, the CarboSource Services, of the Complex Carbohydrate Research Center, University of Georgia, USA (https://www.ccrc.uga.edu/~carbosource/CSS_home.html), PlantProbes, of the University of Leeds, UK (<http://www.plantprobes.net>), Biosupplies Australia (<http://www.biosupplies.com.au>), or the WallBioNet: Plant Cell Wall Biosynthesis Research Network (<http://glycomics.ccrc.uga.edu/wall2/index.html>). mAbs have been utilized to bind epitopes present on RG-I (10), homogalacturonan (11), xylogalacturonan (12), xylans (13), xyloglucan (14), arabinoxylans (13), and arabinogalactan (15). We used a collection of these mAbs in Chapter 3 to study the dynamic fermentation of rapeseed meal fibres by the pig microbiota.

The aim of the study was to create a set of fusion proteins with 35 different CBMs and green fluorescent protein (GFP) as a reporter. This (initial) toolbox with CBM-GFP fusions extends the monoclonal antibodies that we have used in Chapter 3 of this thesis.

Materials and methods

Carbohydrate binding modules, CBMs

Cloning The nucleotide sequence of CBM domains were downloaded from NCBI and the genes were synthesized by BaseClear B.V. (Leiden, The Netherlands). Care was taken that no BamHI, EcoRI or KpnI sites were present in the synthesized genes, as these restriction sites were used for cloning. When these sites were present, the nucleotide sequence was changed such that this did not lead to an amino acid change in the expressed fusion protein. The synthetic genes contained a BamHI or EcoRI site on the 5'-end and a KpnI site on the 3'-end. A list of CBM domains and their (predicted) specificities is shown in Supplementary Table 1. The gene fragments were cloned using standard procedures into pET 28c (containing the green fluorescent protein gene (GFP) with an N-terminal six-histidine tag) using the 5' BamHI or EcoRI and 3' KpnI sites and transformed into *E. coli* DH5 α . The reconstructed expression vectors encode GFP + CBMs fusion proteins (Figure 1). The DNA sequence fidelity of all constructs was verified by bidirectional sequencing by BaseClear.

Plasmids DNA electrophoresis plasmid DNA was isolated from *E. coli* transformants using QIAprep Spin Miniprep Kit (QIAGEN, Venlo, The Netherlands). Restriction enzymes (BamHI and KpnI) were used to cut out the insert fragment in 2 h at 37 °C in 50 μ L of reaction buffer. Then, the fragments size was assessed using the Bioanalyzer Instrument (Agilent, California, United States).

Protein overexpression and purification All verified constructs were transformed into the expression strain, *Escherichia coli* BL 21 (DE3). Next, 250 mL of Luria-Bertani broth, containing 50 μ g/mL kanamycin, was inoculated with the transformed cells and incubated at 37 °C. Once an optical density of 0.6 at 600 nm was reached, expression of the recombination protein was induced by adding isopropyl 1-thio- β -D-galactopyranoside (IPTG) to a final concentration of 1 mM. The cultures were incubated for an additional 5 h at 17 °C. Cells were harvest by centrifugation at 4 000 x g for 20 min. The His-tagged fusion proteins were subsequently purified by using the QIAexpress[®] Ni-NTA Fast Start kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol.

SDS-PAGE and Coomassie Staining The molecular weight of the purified overexpressed proteins was determined the proteins molecular weight using SDS-PAGE according to the manufacturer's protocol (Bio-Rad Laboratories, California, United States). SDS-PAGE gel was stained with Coomassie staining solution (Bio-Rad Laboratories).

Glycome profiling (Glycoprofiling) of polysaccharide panel

Polysaccharides Polysaccharides from various plant sources were obtained from Megazyme (Bray, Wicklow, Ireland) and Sigma (St. Louis, Missouri, United States) (Table S1). Stock solutions were prepared by solubilizing the polysaccharides at 1 mg/mL in deionized water and storing these stock solutions at -20 °C.

Glycoprofiling Samples prepared above were applied (50 μ L of 100 μ g/mL in deionized water per well) in duplicate to Corning[®] 96 well black plates (Corning Inc., New York, United States) and dried to the well surfaces by evaporation overnight at 37 °C. Control wells contained deionized water. The plates were then blocked with 200 μ L of 11 % (w/v) bovine serum albumin (BSA) in Tris-buffered saline (50 mM Tris-HCl, pH 7.6, containing 100 mM sodium chloride) for 1 h. The blocking agent was removed by aspiration, and 50 μ L of purified CBM-GFP fusion proteins were added to each well and incubated for 1 h at room temperature. Subsequently, the supernatant was removed and wells were washed three times with 200 μ L of 0.1% (w/v) BSA in Tris-buffered saline (wash buffer). Next, CBM proteins (50 μ L) were added to a well as positive control (maximum fluorescence). The GFP fluorescence of each

microplate well was subsequently read by using a multi-mode microtiter plate reader (BioTek Synergy HTX, BioSPX, Abcoude, The Netherlands).

Result and discussion

The synthesized CBM genes were inserted into pET28c vector, containing the GFP gene with an N-terminal six-histidine tag (see Figure 1 for the case of CBM27). In total, 35 CBM genes were used to reconstruct fusions with GFP in the expression vector. These were CBM2, CBM3, CBM6, CBM4-9-1, CBM4-9-2, CBM11, CBM13, CBM13-1, CBM13-2, CBM13-3, CBM13-4, CBM15, CBM17, CBM20, CBM21, CBM26, CBM27, CBM29-65-1, CBM29-65-2, CBM35, CBM36, CBM39, CBM41, CBM47, CBM51, CBM58, CBM60, CBM61, CBM62, CBM63, CBM66, CBM67, CBM71, CBM80 and CBM81 (Supplementary Table 1). The sizes of the inserted fragments in the plasmids were assessed by electrophoresis, which showed that the insert size of each CBM was consistent with their gene size (for an example see Figure 2). Constructs with the correct size were subsequently verified by bidirectional sequencing. The results of SDS-PAGE of the Ni-column purified proteins (Figure 3) also revealed that the protein molecular weight of each CBM-GFP fusion was in line with their predicted size (using their gene maps).

The key steps of the Glycoprofiling technique are summarized in Figure 4 and can be divided into three stages: polysaccharide immobilization, epitope recognition by CBM and binding of the CB-GFP fusion protein, and fluorescence reading. The type of 96-well plate was selected based on literature (16), where the polysaccharides can optimally immobilize on the surface of the bottom of the plate.

The reproducibility of the Glycoprofiling technique was tested by comparing data derived from duplicated wells. The results shown in Table 1 indicate that CBM27, which has the specificity to recognize α -D-(1-6)-Galp and β -D-(1-4)-Manp, binds to xylan (beechwood), galactomannan (carob), galactomannan, guar galactomannan, D-galacto-D-mannan, and locust bean gum. The coefficient of variation (CV) of fluorescence values for these polysaccharides were 7.9%, 10.9%, 5.2%, 8.3%, 3.4%, and 5.7%, respectively. The low CV values indicated high reproducibility. Furthermore, the rest of the 35 polysaccharides showed no binding, which indicated the high specificity of the CBMs (Table S2). The maximum fluorescence signal for CBM27 was on average 20.5 (Table S1, positive control), indicating particularly strong binding of CBM27 to D-galacto-D-mannan (same average fluorescence signal as for positive control; Table 1). Although it is not possible to conclude this for all fusion proteins, the fact that the CBM27-GFP fusion still recognizes its (predicted) target, shows that at least this fusion protein correctly folds into its separate domains (GFP and CBM). Care was taken in constructing the fusion proteins to include a few amino acids more than just the CBM domain, which could function as a sort of linker between the two domains in the fusion. Future analysis will have to show whether the other fusion proteins are also correctly folded. In any case, all fusion proteins fluoresced green upon excitation (data not shown) suggesting correct folding of the other fusion proteins as well.

When used on plant material (such as rapeseed meal), the profiling results would provide a global overview of the cell wall polysaccharide composition and structure. The toolbox can be used to extend the monoclonal antibody repertoire that we used in Chapter 3, and can easily be extended with additional CBM-fusion proteins. Moreover, fusion proteins with different colors (e.g. m-Cherry (red), YFP (yellow) or BFP (blue)) can be made by simple exchange with GFP. This toolbox may become essential in the study of the dynamics of breakdown of dietary fibres by the (pig) gut microbiota.

Figure 1. An example of plasmid gene map (CBM27).

Figure 2. CBM-Plasmid DNA electrophoretic image in the Bioanalyzer for 12 representative CBM constructs after digesting with BamHI and KpnI. The black colored-bands are the vector, the light-grey colored bands of different size in each lane are the respective CBM genes. The blue and green colored bands are the internal standards for the Bioanalyzer.

Figure 3. SDS-PAGE analysis on the expression of representative recombinant CBM-GFP fusionis. Multiple lanes with the same CBM-code indicate separate eluted fractions collected from the Ni-column.

Figure 4. Schematic representation of fluorescence reading in Glycoprofiling.

Table 1. Fluorescence values due to binding of CBM27 to polysaccharides when tested in the Glycoprofiling.

	CBM27-1	CBM27-2	C.V. %
Guar medium viscosity xylan(beechwood)	17	19	7.9
Galactomannan(carob) high viscosity	14	12	10.9
Guar galactomannan(GD21) enzyme modified guar	14	13	5.2
Guar galactomannan (GD 28) enzyme modified guar	18	16	8.3
D-galacto-D-mannan from ceratonia	21	20	3.4
Locust beangum from ceratonia siliqua seeds	13	12	5.7

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Supplemental Table:

Table S1. A list of the (predicted) specificities of CBMs

CBMs	Specificity
CBM2	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp
CBM3	β -D-Glcp-(1-4)- β -D-Glcp
CBM6	β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp
	β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp
	β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp
	β -D-Glcp-(1-4)- β -D-Galp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp
CBM4-9-1	β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp
CBM4-9-2	β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp
CBM11	Beta-1,4- and Beta-1,3-1,4-Mixed Linked Glucans at a Single Binding Site
CBM13	α -D-Manp-(1-2)- α -D-Manp
	α -D-Manp-(1-2)- α -D-Manp
	α -D-Manp-(1-2)- α -D-Manp
	α -D-Manp-(1-2)- α -D-Manp
	α -D-Manp-(1-2)- α -D-Manp-(1-2)- α -D-Manp
CBM13-1	α -L-Arap
	β -D-Galp
CBM13-2	β -D-Galp-(1-4)- β -D-Glcp
	β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- α -D-Xylp
	β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp
CBM13-3	β -D-Galp-(1-4)- α -D-Glcp
CBM13-4	β -D-Galp-(1-3)- β -D-Galp
	β -D-Galp-(1-3)- β -D-Galp-(1-3)- β -D-Galp
	β -D-Galp-(1-4)- β -D-Glcp
	β -D-Galp
	β -D-Galp1S-(1-1)-<non carb>
CBM15	β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp-(1-4)- β -D-Xylp
CBM17	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp
CBM20	α -D-Glcp-(1-4)- α -D-Glcp
	α -D-Glcp-(1-4)- α -D-Glcp-(1-4)- α -D-Glcp1N-(1-4)- α -D-6-deoxy-Glcp4N-(1-4)- α -D-Glcp-(1-4)- β -D-Glcp
	α -D-Glcp-(1-4)- α -D-Glcp
	α -D-Glcp-(1-4)- β -D-Glcp
CBM21	α -D-Glcp-(1-6)- α -D-Glcp-(1-6)- α -D-Glcp-(1-6)- α -D-Glcp
CBM26	α -D-Glcp-(1-6)- α -D-Glcp-(1-6)- α -D-Glcp
	β -D-Glcp
CBM27	β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
	α -D-Galp-(1-6)[β -D-Manp-(1-4)]- β -D-Manp-(1-4)[α -D-Galp-(1-6)]- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
CBM29- CBM65-1	
CBM29- CBM65-2	β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- α -D-Glcp
	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- α -D-Glcp
	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- α -D-Glcp
	β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
CBM35	α -D-Glcp-(1-6)- β -D-Glcp
	α -D-Glcp-(1-6)- α -D-Glcp
	α -D-Glcp-(1-6)- α -D-Glcp-(1-4)- α -D-Glcp
CBM36	β -D-Xylp-(1-4)- β -D-Xylp-(1-1)-methyl
CBM39	β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp-(1-3)- β -D-Glcp
CBM41	α -D-Glcp-(1-4)- α -D-Glcp

	α -D-Glcp-(1-4)- α -D-Glcp
	α -D-Glcp-(1-4)- α -D-Glcp-(1-4)- α -D-Glcp
	α -D-Glcp-(1-4)- α -D-Glcp
	α -D-Glcp-(1-4)- α -D-Glcp-(1-4)- α -D-Glcp-(1-4)- α -D-Glcp
CBM47	α -D-Glcp-(1-3)- α -D-Glcp
	α -D-Glcp-(1-4)- β -D-Glcp
CBM51	β -D-Galp-(1-1)-methyl
	<non carb>-(1-4)- α -D-6-deoxy-Glcp4N-(1-4)- α -D-Glcp-(1-4)- α -D-Glcp
	<non carb>-(1-4)- α -D-6-deoxy-Glcp4N-(1-4)- β -D-Glcp-(1-4)-ASP
CBM58	α -D-Glcp-(1-4)- α -D-Glcp
	β -D-Glcp-(1-4)- β -D-Glcp
CBM60	β -D-Galp-(1-4)- β -D-Galp
	β -D-Galp-(1-4)- β -D-Galp-(1-4)- β -D-Galp
CBM61	β -D-Galp-(1-4)- β -D-Galp-(1-4)- β -D-Galp
CBM62	α -D-Galp-(1-6)- β -D-Manp
	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp
CBM63	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp
	β -D-Fruf
CBM66	β -D-Fruf-(2-6)- β -D-Fruf
CBM67	α -L-Rhap
	β -D-Galp-(1-4)- β -D-GlcpNAc
	β -D-Galp-(1-2)- α -D-Xylp-(1-6)[β -D-Glcp-(1-4)]- β -D-Glcp-(1-4)- β -D-Glcp
CBM71	α -D-Galp-(1-6)- β -D-Manp
	β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
	β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp
	β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
CBM80	β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp-(1-4)- β -D-Manp
	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp
CBM81	β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp-(1-4)- β -D-Glcp

Table S2. Duplicate fluorescence values due to binding of CBM27-GFP fusion to polysaccharides when tested in the Glycoprofiling.

	CBM27	CBM27
Wheat arabinoxylan insoluble	0	0
Wheat arabinoxylan acid debranched; 22% arabinose	0	1
1,4- β -D-mannan Brohydride reduced	0	0
Mannan(Ivory nut)	0	0
Wheat arabinoxylan low viscosity	0	0
Galactomannan(carob) low viscosity	2	4
Debranched arabinan	0	0
Azo-galactomannan from carob	2	4
Lichenan (Icelandic moss) 1,3:1,4- β -D-Glucan	0	1
Rye arabinoxylan high viscosity	0	0
Pullulan	0	0
Guar medium viscosity xylan(beechwood)	17	19
Karaya gum	0	0
Rhamnogalacturonan from soy bean pectic fibre	0	0
Larch arabinogalactan	0	1
Galactomannan(carob) high viscosity	14	12
Guar galactomannan(GD21) enzyme modified guar	14	13
Guar galactomannan (GD 28) enzyme modified guar	18	16
Arabinan sugar beet	0	0
Pectic galactan potato	0	0
Beta glucan(barley) low viscosity	0	1
Linear 1,5- α -L-arabian sugar beet	0	1
Galactan	0	1
Pachyman 1,3- β -D-glucan	0	0
Xyloglucan from tamarind seed	0	0
Cm-pachyman	0	0
Rhamnogalacturonan from potato pectic fibre	0	1
Cm-curdlan degree of carboxymethylation	0	0
Amylose (potato)	0	0
Glucomannan(konjac) low viscosity	5	5
Chitin from shrimp shells	0	0
D-galacto-D-mannan from ceratonia	21	20
Dextran	0	0
Cm-cellulose 4M for endo-cellulase	0	0
Xanthan gum from axanthomonas	0	1
Tragacanth	0	2
Gum Arabic	0	0
4-O-Methyl-D-glucurono-D-xylan	0	0
Xylan beechwood	0	0
Locust beangum from ceratonia siliqua seeds	13	12
Beta glucan (oat) medium viscosity	0	2
Negative control	0	1
Positive control	20	21

Chapter 7

General discussion

7.1 Motivation and aim of the research

The project titled “Improving intestinal degradation of recalcitrant fibres in pigs” aimed to improve the utilization of recalcitrant fibres in swine feed from by-products of seed oil industry, in order to reduce feed costs and maintain sustainable use of feed raw materials.

As fibres are fermented/utilized by the gut microbiota, a swine large intestinal *in vitro* model was developed to study the effect of fibres on swine gut microbiota. In this thesis, fibres in rapeseed meal (RSM), which is a cheap and abundant by-product of rapeseed oil production, were studied. The effect of three enzymatic treatments and one chemical treatment on the recalcitrant fibres of RSM was investigated. The effect of the unmodified and the four modified RSMs on the swine gut microbiota (composition and activity) was studied. Last, a preliminary novel high-throughput technique to screen polysaccharide composition and structure was developed.

7.2 Swine *in vitro* Large Intestinal Model

The Swine *in vitro* Large Intestinal Model (SLIM) was developed based on the human, computer-controlled, dynamic TNO *in vitro* model of colon, nick-named TIM-2 (1). As TIM-2 was originally designed for human beings, the colon parameters, such as temperature (37 °C), pH (5.8), and Standard Ileal Efflux Medium (SIEM), are adapted to the physiology of human beings. For SLIM, therefore temperature (39 °C), pH (5.9) and medium composition (SIEMP; SIEM for pigs) were modified to correspond to swine physiology (2, 3)

7.2.1 Similarities between the microbiota composition of the original pig fecal inoculum and the microbiota fed with modified SIEM containing different starch sources

After feeding the gut microbiota in SLIM modified SIEM containing different starch sources (maize, potato or wheat), in search of the optimal starch source for the pig microbiota, the microbiota composition was determined and a network analysis was performed (Chapter 2). The different starch sources impacted the microbiota composition: they had a considerable effect on both alpha-diversity (inverse Simpson, and Faith’s PD), and beta-diversity (weighted and unweighted UniFrac, and Bray-Curtis) (Chapter 2), indicating that some microbial species (composition and/or abundance) among the three starch treatments were significantly different. In Chapter 2, among the three starch treatments, 11 genera, 7 genera and 7 genera were more abundant upon maize, potato, and wheat starch treatment, respectively. The inherent properties of starches, such as crystallinity, granular structure, amylose:amylopectin ratio, and retrogradation of amylose, will influence the formation of resistant starch (4). Therefore, the proportion and amount of resistant starch types of the starch sources may have led to alteration of the microbiota composition after feeding these starches.

The ensemble method was used based on multiple similarity measures in combination with generalized boosted linear models on microbial abundance (5). This investigates whether the shift of microbial abundance will change the bacterial co-occurrence relationships. Our results showed that maize starch-based network had much better scale-free properties, as evaluated by the value of degree distribution fitting R^2 (Chapter 2). Scale-free networks display a very strong robustness against random failures (6). Thus the microbiota fed with maize starch may be more stable or resilient, compared to those fed with potato and wheat starch.

In order to investigate which functional composition driven by the different starch sources was more similar to the original pig microbiota inoculum, a pig fecal sample was included in the analyses. Both the PCA and the heatmap of the functional predictive profile showed that

the original pig fecal sample clustered with maize starch treatment, which indicated that the functional profile of these samples was more similar to the original pig inoculum (Chapter 2).

As discussed above, the microbiota fed with maize starch may be more stable or resilient, compared to those fed potato or wheat starch, which can more closely simulate the microbial ecology *in vivo*. And the predictive functional metagenome of the microbiota fed with maize starch was more similar to the original inoculum. Thus, maize starch was used as starch source in the recipe of standard ileal effluent medium for pigs (SIEMP). But the microbial composition between microbiota fed with maize starch and the original inoculum still was very different. The original inoculum separated in a principal coordinate analysis from the microbiota fed with one of the three starch sources, which indicated a different microbial composition between the original inoculum and starch-fed microbiotas (Chapter 2). This is reasonable, as pig diets have different carbohydrate sources than human beings. The dominant phyla in the maize starch-fed microbiota were Firmicutes and Actinobacteria, while they were Firmicutes and Bacteroidetes in the original inoculum (Chapter 2). Previous reports (7-9) have also shown that the most abundant phyla were Firmicutes and Bacteroidetes in pig gut microbiota. Therefore, the next study was performed, in which an attempt was made to adjust the microbial composition in the *in vitro* model to become more similar to the original inoculum.

7.2.2 Similarities between the microbiota composition of the original pig fecal inoculum and the microbiota fed with maize starch-based SIEMP supplemented with arabinoxylan (AX) or AX and xyloglucan (XG)

Compared to only supplementing AX to maize starch based SIEMP, microbiota fed AX-XG on top of maize-starch had a higher relative abundance of Bacteroidetes and Firmicutes, and a lower relative abundance of Actinobacteria, which made the microbial composition more similar to the original inoculum (Chapter 2). This indicated that the XG supplementation can further increase a relative abundance of bacteria in the phyla Bacteroidetes and Firmicutes, at the expense of the phylum Actinobacteria. Larsbrink et. al (10) confirmed Bacteroidetes can catabolize XG by a discrete genetic locus, which corroborates that XG supplementation induced the growth of Bacteroidetes in our study. Cantu-Jungles et. al (11) reported that a substantial increase in the *B. uniformis* population occurred after XG addition to an *in vitro* fecal fermentation. At a lower phylogenetic level in our study, XG mainly increased the amount of Prevotellaceae, and led to a slight decrease in the number of Atopobiaceae, Lachnospiraceae, and Muribaculaceae. We did not find any research about Prevotellaceae catabolizing XG in pigs. But Svartström et. al (12) reported that Prevotellaceae from the rumen microbiota can express xyloglucanases, which are XG-degrading enzymes.

Supplementation with both AX and XG in maize-based SIEMP also showed higher reproducibility than only supplementing AX to maize-based SIEMP. Chapter 2 shows that microbiota compositions between replicates at different time points upon AX-XG feeding was much more similar than those on AX. In order to test the reproducibility of microbial composition, C.V. of replicates of microbiota fed with AX-XG of alpha diversity, microbial abundance, and microbial activities were evaluated (Chapter 2), which were all lower than 15% (except for 5 taxa in microbial abundance). The low C.V.s indicated that the results of the biological replicates were very reproducible (13). The high reproducibility of TIM-2 (the human version) was previously evaluated by the clustering of the microbiota after the adaptation period (at t0) (14), and during an entire experiment (15). Since SLIM (or TIM-2)

is computer-controlled, it is highly reproducible. This has been shown in numerous studies (15-18).

All data collectively considered, maize starch with arabinoxylans (AX) and xyloglucan (XG) should be added to the SIEM recipe to simulate ileal effluent medium for pigs (SIEMP), and this outcome laid the foundation for experiments mimicking the swine large intestine fermentation in the other chapters of this thesis.

7.3 Characteristics of non-processed and processed RSM

RSM was processed by a cellulase (CELL), two pectinase (PECT1 and PECT2), alkaline (ALK) treatment or nothing (CON) by two different method (Figure 1). Method I, RSM was processed before predigesting its protein; Method II, RSM was processed after predigesting its protein (Figure 1). Moreover, biological (Chapter 3) and chemical method (Chapter 5) were performed to characterise polysaccharide composition of non-processed and processed RSM.

Figure 1. Schematic representation of processing RSM by two different method.

7.3.1 Evaluation by glycoprofiling with monoclonal antibodies (mAbs)

Cell wall polysaccharides of RSM (*Brassica napus*) were comprehensively studied by chemical methods (19), which showed that they generally consisted of arabinan, homogalacturonan, Rhamnogalacturoan I (RG-I), type II arabinogalactan (AG), and xyloglucan (XG) (20). In a previous study (Long, de Vries and Venema submitted), we determined the monosaccharide constituent composition, which was in line with Pustjens et al. (2013). Our current findings, using glycoprofiling with the mAbs, are also in line with this, since nonfucosylated XG-, RG-I-, pectic backbone-, and AG-recognizing mAbs bound to CON. By similar reasoning, xylan was also detected in CON, which was not reported before, although it is not entirely clear how much cross-reactivity the mAbs show.

ALK treatment intensively increased binding of mAbs that specifically bind to nonfucosylated XG (Chapter 3). This is consistent with previous finding that alkali could extract XG from hemicellulose (19), which led to XG being detected by the mAbs in the current study. Previous research also demonstrated that XG is linked via hydrogen bridges to the surface of cellulose microfibrils, which is extractable with alkali, but not accessible by enzyme (21). However, enzymatic treatments (PECT1, PECT2, and CELL) led to a disappearance of binding nonfucosylated XG-specific mAbs compared to CON, which was unexpected. It could be that XG might become entrapped by other cell wall structures after pectinase or cellulase treatment. It is unknown whether this kind of the entrapment phenomenon would reduce the degradability of XG.

Enzymatic treatments increased binding of linseed mucilage RG-I-specific mAbs and some mAbs directed against the arabinogalactan side chains of RG-I (RG-I/AG), and reduced binding of some mAbs directed to pectic backbone, which indicated that PECT1 and PECT2 broke down pectic backbone and exposed RG-I and its arabinogalactan side chains. These results are supported by a previous study showing that PECT1 and PECT2 degraded pectic

backbone and released RG-I and its arabinogalactan side chains (19). Moreover, RG-I attached to cellulose microfibrils can be released by cellulase (22), while at the same time it might block the accessibility of pectic backbone because of the shifting of the polysaccharides structure.

ALK treatment led to the disappearance of binding of mAbs that specifically bind to AG-1 while it increased binding of mAbs that specifically binding to AG-2, which indicated that ALK could cause arabinogalactan to be physically entrapped in other cell wall structures, while linear and branched arabinan become accessible (23).

Their binding strengths of mAbs in AG-4 were lower upon ALK_B, PECT1_B, PECT2_B, and CELL_B treatment, compared to ALK_A, PECT1_A, PECT2_A, and CELL_A. This indicated that small fragments were produced in AG-4 group after enzymatic and chemical treating RSM, which were dialyzed out in ALK_B, PECT1_B, PECT2_B, and CELL_B during the subsequent predigestion treatment. Overall, both enzymatic and chemical processed RSM can release some polysaccharides but physically entrap or shield some others at the same time.

7.3.2 Evaluation by chemical method

The carbohydrate content of CON is 62% w/w (Chapter 5). Dominant sugars are glucose (Glc, 31 mol %), uronic acid (UA, 19 mol %), galactose (Gal, 11 mol %) and arabinose (Ara, 5 mol %). This is inline with previous study (19). CON contained 56 % pectin (defined as: rhamnose + arabinose + galactose + uronic acid) and 44 % (hemi)cellulose (xylose + mannose + fucose + glucose).

The values of pectin and (hemi)cellulose for ALK_A, ALK_B, PECT1_A, PECT1_B, PECT2_A, PECT2_B, CELL_A, and CELL_B were 55 % and 45%, 52 % and 48%, 54 % and 46 %, 50 % and 50%, 56 % and 44 %, 51 % and 49 %, 57 % and 43 %, and 60 % and 40 %, respectively. Relative pectin contents decreased in ALK, PECT1, and PECT2 treatment, as expected for the PECT enzymes, while they increased in CELL treatment, compared to CON. PECT1 and PECT2 did not decrease pectin to a large extent, which might be caused by shielding or inaccessibility of the pectin molecules by (hemi)cellulose.

The ALK, PECT1, and PECT2 treatments increased (hemi)cellulose values, compared to CON, as expected. Predigesting before or after processing RSM had little effect on the monosaccharide constitution.

7.4 The effect of (processed) RSM on the microbiota composition and fibre degradability in the SLIM

In SLIM, the swine microbiota was fed with non-processed and processed RSM by a single shot of 5 g RSM (Chapter 3, Figure 2A), continuously 48 h feeding of RSM (Chapter 4, Figure 2B), or a single shot of 5 g RSM after 48h of adaptation to RSM (Chapter 5, Figure 2C). In order to investigate the effect of processed RSM on the microbiota, β -diversity and relative abundance of microbial genera and predictive microbial function profile were analyzed.

Figure 2. Schematic experimental setup for fibre fermentation in the Swine Large Intestine in vitro model (SLIM), in Chapter 3 (A), Chapter 4 (B) and Chapter 5 (C).

7.4.1 Gut microbiota

Our studies suggested that processed RSM (ALK, PECT1, PECT2 and CELL) had a significant different microbial community compared to CON as evaluated by unweighted UniFrac, no matter how the RSM was fed (Chapter 3, 4 and 5). Dietary fibre is known to have a considerable effect on gut microbiota composition (24-26). Characterization of non-processed and processed RSM showed that polysaccharide structures were differentially shifted due to ALK, PECT1, PECT2, and CELL treatment in Chapter 3 and 5, which were consistent with previous studies (27, 28). These observations might explain the specific genus changes in microbiota composition after microbiotas were fed with ALK, PECT1, PECT2, and CELL compare to CON (Chapter 3, 4 and 5), despite the differential effect of the treatments on RSM as assessed by biological and chemical method (Chapter 3 and 5).

In order to understand which microbes were significantly influenced by dietary supplementation of processed RSM, microbial genera determined by next generation sequencing (V3-V4 region of the 16S rDNA gene) were compared among the five treatments. In Chapter 3 (Figure 2A), the relative abundances of *Ruminococcaceae NK4A214* group, *Ruminococcaceae UCG-002*, *Ruminococcaceae UCG-005*, *Roseburia*, *Bifidobacterium*, and *Christensenellaceae R-7* group were significantly increased in ALK, PECT1, PECT2, and CELL. Research has shown that genera of the family *Ruminococcaceae* contain major (hemi)cellulolytic and pectinolytic species (29-31). Thus, microbes from the family

Ruminococcaceae play an important role in degrading (hemi)cellulose and pectin in their activity against recalcitrant fibre (32, 33). Previous research demonstrated that the higher the relative abundance of *Ruminococcaceae* NK4A214 group, the higher the fibre degradability (30, 34). This observation was consistent with the current study, which showed that the SCFA production (which is usually used as evaluation of fibre degradability *in vitro*) was also higher in ALK, CELL, PECT1, and PECT2 compared to CON. Furthermore, our study also showed that the relative abundance of *Ruminococcaceae* NK4A214 group, as well as *Ruminococcaceae* UCG-002 and *Ruminococcaceae* UCG-005, significantly correlated with acetic acid, propionic acid, butyric acid, and (not surprisingly) total SCFA production. *Roseburia* is a well-known butyrate-producing bacteria (35), and a primary degrader of β -mannans (36). The significant increase in abundance of this genus was accordance with the increased butyric acid production in Chapter 3, and increased predicted abundance of beta-mannosidases (EC.3.2.1.25) in the processed RSM groups. *Bifidobacterium* is suggested to be a common cross-feeding bacteria for sugar utilization (37). Previous research has been shown that *Bifidobacterium bifidum* relies on the presence of a primary degrader in order to grow with either resistant starch or xylan both *in vitro* (38) and *in vivo* (39). It has also been reported that numerous *Bifidobacterium* species grew to higher cell densities accompanied by upregulating their respective saccharolytic pathways when grown in co-culture compared to their growth in monoculture (40). This finding was consistent with the current study, where the relative abundance of *Bifidobacterium* was much higher than that of other genera, especially in ALK. Alternatively, synergy existed among genera that were targeting the same substrates, possibly by specializing in degrading different motifs within the fibre molecule. The relative abundance of *Christensenellaceae* R-7 group increased after enzymatic and chemical treatment, which was consistent with a previous study where rumen microbiota was fed with fibrolytic enzyme-treated wheat straw (41). *Christensenellaceae* plays an important role in degrading fibre (42) and producing acetic and butyric acid (43). All the observations above were supported by former reports that feed processed by carbohydrase enzymes could stimulate growth of specific microbes (44-46). It can be speculated that the pretreatment of feed stuffs with carbohydrases causes the release of hydrolysis materials (presumably oligosaccharides), which promote the chemotactic response of specific bacteria, and stimulates their attachment to feed particles, thereby leading to growth of these microbes (45, 47-49).

In the Chapter 5 (Figure 2C), CELL treatment of RSM significantly increased the numbers of *Olsenella*, [*Eubacterium*] *nodatum* group, *Acidaminococcus*, *Lachnospiraceae* NK3A20 group, *Bifidobacterium*, *Acetitomaculum*, and *Syntrophococcus*. These genera may prefer to utilize cellulose- and/or hemicellulose-fragments generated by the action of CELL. However, in the literature only *Eubacterium* has been reported as cellulolytic (50). The CELL enzyme preparation contains multiple glycolytic activities, including exo-1,4- β -glucanase (cellobiohydrolase), endo-1,4- β -glucanase, hemicellulase and β -glucosidase. CELL may have broken down some bonds in cellulose thereby enhancing (hemi)cellulose utilization by the gut bacteria and at the same time also expose other polysaccharides (e.g. pectins) to other bacteria. ALK increased *Megasphaera*, and *Ruminococcus gauvreauii* group. Reports show that *Ruminococcus* are the most commonly found cellulolytic organisms (32, 50-52). *Megasphaera* is reported to contain glycosyl hydrolase (GH) family 53, which is involved in plant cell wall degradation (53). These reports together with our results indicate that ALK can disrupt the cell wall architecture, by solubilization of polysaccharides, presumably by breaking hydrogen bonds and hydrolyzing ester linkages thereby removing esters present as

decoration on polysaccharides, making them more accessible for further enzyme degradation and utilization by the microbiota. Interestingly, both PECT1 and PECT2 showed no significant difference in Shannon index compared to CON and a very similar microbiota composition, which seemed to indicate that neither pectinase really changed the cell wall structure, or the changes did not meet the swine gut microbiotas' hydrolytic capacities, and as a result no bacteria were selectively stimulated. We reported before that a cocktail of PECT1 and PECT2 improved degradability of non-glucose polysaccharides of RSM in broilers (27, 28). Nevertheless, it seems that cell wall break down by PECT1 and PECT2 does not lead to advantages for members of the swine gut microbiota.

7.4.2 Fibre degradation

To investigate the dynamic changes of polysaccharide composition and structure in CON, ALK, CELL, PECT1, and PECT2 treated RSM during *in vitro* fermentation, a time series of sampling was performed and the set of 34 mAbs was used to screen the lumen digests. Glycome profiling showed that binding of mAbs to lumen digests were dynamic during the *in vitro* fermentation in SLIM (Chapter 3). No bindings of mAbs recognizing non-fucosylated XG were observed in lumen samples after the microbiota was fed with ALK or CON (Chapter 3), which was unexpected as binding signals were seen in the substrates themselves prior to addition to SLIM (Chapter 3). The hypothesis could be entertained that XG was immediately utilized by bacteria before our first sampling time point (after 30 minutes), or that the XG structures were unable to be recognized by the mAbs after supplementing them to lumen, due to entrapment by other molecules. Binding signals still existed at t24 for all mAbs that showed signal at the t0.5 time point, which indicated that these structures cannot be degraded any further or more fermentation time was needed. Glycome profiling showed that binding of mAbs recognizing each polysaccharide structure/epitope were dynamic during the 24 h fermentation period (Chapter 3). It is not unlikely that certain polysaccharide structure, such as (hemi)cellulose and pectin, were exposed to microbes stage by stage, due to ever increasing degradation of the cell wall structures over time. For instance, bacteria should break down side-chain AG before they can utilize the backbone of RG-I. However, this hypothesis should be validated in future studies.

The chemical analytical method (Chapter 5) showed that the amount of main monosaccharides (arabinose, galactose, glucose, and uronic acid) was lower after 24 h fermentation for ALK and CELL compared to CON. The utilization of the main monosaccharides plateaued after time point 52 (4 hours after the shot), except for glucose, which was continuously utilized until time point 72 (24 hours after the shot). The utilization of arabinose and galactose was faster in ALK and PECT1, compared to the other treatments. HPSEC showed that an almost complete disappearance in the high Mw fraction occurred from 4 h onward (at time point 52 h), indicating degradation and/or utilization of all high Mw polysaccharides. Compared to the highest peak of the elution profile of CON (48.5 h), the highest peaks of the elution profile of ALK and CELL (50 h and 49 h, respectively) showed a higher amount of high Mw material, while those of the elution profile of PECT1 and PECT2 were approximately equal to CON (Chapter 5). This indicated that more materials were solubilized under the conditions when ALK and CELL-treated RSM were fed, than with the other substrates.

SCFA production was used to evaluate the fibre degradability of RSM by the swine gut microbiota. The cumulative acetic, propionic, and butyric acid and total SCFA production were higher in ALK, CELL, PECT1, and PECT2 compared to CON after the single shot (Chapter 3; Figure 2A). This observation was consistent with the predicted functional profiles

related to carbohydrate metabolism, where the relative abundance of fibre breakdown and fermentation enzymes and pathways increased (Chapter 3). This finding was in accordance with previous studies that addition of pectolytic enzymes improved degradability of non-starch polysaccharides (NSP) of RSM *in vitro* (27) and in broilers (28, 54). Giraldo et al. also reported that supplementation of exogenous cellulase increased SCFA production (45). Previous research demonstrated that supplementation of carbohydrase to feed before feeding could increase microbial protein production, ruminal cellulolytic bacterial numbers, and ruminal fibrolytic activity (44). Thus, the findings above indicate that the enzymatic and chemical treatment on RSM could sufficiently open cell wall architecture, to enable effective accessibility of NSP to bacterial degradation enzymes, and subsequently stimulate expression of microbial saccharolytic pathways.

In Chapter 4 (Figure 2B), there were no significant differences in SCFA production between unprocessed and processed RSM during the fibre fermentation period. This indicated that the processing applied to RSM could not improve their degradability compared to CON, in other words, they might not increase the feed efficiency during this period *in vivo*. It might be the fibre adaptation period was not enough to express the maximum enzymatic potential (55). Alternatively, the 48 h fibre adaptation period was sufficient for the microbiota to allow it to adapt to the substrate and even ferment CON efficiently.

The amount of propionic acid and the total SCFA in ALK and CELL were significant higher than those in CON, while there were no significant differences in SCFA production in PECT1 and PECT2 compared with CON in Chapter 5 (Figure 2C). This was corroborated by the carbohydrate related microbial functional metabolism pathways, which showed that the abundances of pyruvate metabolism, propanoate metabolism, pentose phosphate pathway, galactose metabolism, energy metabolism, fructose and mannose metabolism, and carbohydrate digestion and absorption were higher in CELL and/or ALK compared to CON. The mobile nylon bag technique was used to validate the fibre degradability in ileal cannulated pigs. The results showed that more fibres were degraded in case of ALK and CELL treatment, compared to CON, while there was no increased fibre degradability in PECT1 and PECT2 treated RSM, compared to CON (Chapter 5).

All data collectively considered, different feed methods (Figure 2A-C) lead to different SCFA production in terms of ALK, CELL, CON, PECT1, and PECT2 (Chapter 3, 4, and 5). A relative long adaptation period is needed after a diet change to RSM for swine microbiota (Chapter 4). Therefore, a gradual feeding of (modified) RSM to the swine gut microbiota might lead to better RSM adaptation. ALK and CELL treatment on RSM can improve its fibre degradability (Chapter 5).

7.5 A preliminary novel high-throughput technique to screen cell wall polysaccharide structures

The study was performed to develop a methodology based on carbohydrate binding modules (CBM), which enables to detect and determine plant cell wall structures in plant materials. The key steps of the Glycoprofiling technique can be divided into three stages: polysaccharide immobilization, CBM recognition and CBM-GFP fusion protein binding, and fluorescence reading. The optimized type of 96-well plate was selected based on literature (23), where the polysaccharides are immobilized well on the surface of the bottom of the plate. The results showed high reproducibility and specificity in recognizing polysaccharides by the Glycoprofiling technique. The set of different GFP-CBM fusion proteins can be extended by adding different CBMs, as well as exchanging GFP for other colored-fluorescent proteins. This would ultimately lead to a suit of fluorescent fusion proteins to dissect the

structure of dietary carbohydrates.

7.6 Future perspectives

The current studies were focused on investigating the effect of modified (enzymatic and chemical treatment) RSM on swine gut microbiota composition and activity, and the fibre degradability by the gut microbiota, with the aim to increase use of recalcitrant fibres by the pig gut microbiota. Our *in vivo* study validated that CELL and ALK rather than PECT1 or PECT2 increased fibre degradation in RSM, which could help to guide feed additive strategies to improve efficiency and productivity in swine industry. The current studies gave insight into how feed enzymes may modulate microbial status, which provides good opportunity to develop novel carbohydrase, particularly in swine feed.

It is complicated and costly to investigate the effect of feed fermentation by the gut microbiota in pigs *in vivo*, due to the interaction with other feed-components in the swine feed (such as other fibers), the ethical considerations (such as using pathogens, toxic compounds, and experiments in animal themselves) and other uncontrollable factors (such as infectious disease or death during the experiment, which are important for reproducibility of results). Therefore, the newly developed SLIM (Chapter 2) is an excellent alternative to study the effect on the composition and/or activity of the gut microbiota without ethical constraints. It allows the screening of a large number of substances such as dietary ingredients and drugs, and even would allow the use of radioactive compounds or pathogens.

The microbes modulated by (processed) RSM, which had significant positive correlations with SCFA production, might be potential SCFA producer or RSM consumer (Chapter 3, 4 and 5). It would be interesting to investigate or validate the role of the potential SCFA producer in improving recalcitrant fibre degradability in a future study. One possible way is to deep mine the genomes of these microbes for encoded the carbohydrases by bioinformatic tools. What is more, these enzymes can be overexpressed in an expression host and subsequently used to process RSM to investigate its degradability in pigs. Our study also showed that some carbohydrate structures (e.g. pectin) could not be accessed by the corresponding enzymes (e.g. pectinase), which is the limit step of improving its degradability. New bioinformatic tools should be developed to explore this kind of new carbohydrase (which can access and degrade the inaccessible structures), which might only exist in specific strains, strains that are increased by these recalcitrant fibres.

The microbes, which significantly negatively correlated with bindings of mAbs, might be the mAbs-specific polysaccharide-epitope degraders (Chapter 3). However, we still do not know fully how the enzymatic and chemical treatment on the RSM cell wall structure affect the bacterial utilization. Thus, known polysaccharide structures, either changed by modification by the feed/microbial enzymes or not, should be fed to the swine gut microbiota to study the limiting step of the utilization of the recalcitrant RSM fibres, in order to develop novel feed enzymes and to determine structure(fibre)-function(microbiota) relationships and the correlation to health. The latter (health status) of course cannot be tested in SLIM, and requires well-controlled animal studies. To truly be able to make such structure-function relationships, more powerful and high-throughput techniques should be developed to analyze the plant cell wall polysaccharides, also dynamically during microbial breakdown. Our glycoprofiling method (Chapter 6) is a promising high-throughput method to screen polysaccharide structures, however, it is still in its preliminary stage, and one has to extend the CBM toolkit. Furthermore, isolation of microbial enzymes, which can significantly utilize the recalcitrant fibre, as eluded to above, might be another way to develop novel cell wall degrading enzymes.

From Chapter 3, PECT1 and PECT2 can improve SCFA production and only ALK and CELL increased fibre degradability as validated in Chapter 5. Therefore, combined addition of pectinase and cellulase/alkaline treatment might further improve the fibre fermentability of RSM. Chapter 4 demonstrated that a relative long adaptation period is needed after a diet change to RSM for the swine microbiota, which has to be considered in practice, in order to improve its fibre utilization.

In conclusion, this thesis showed the initial steps for enzymatic and chemical treatment of RSM to significantly improve its fibre fermentability(*in vitro* and *in vivo*) and change microbiota composition *in vitro*. The combination of both pectinase and cellulase is expected to further improve recalcitrant fibre degradability. The potential SCFA producers and polysaccharide degraders in RSM utilization might be worthy of studying further for their importance in improving RSM fibre fermentability.

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Summary

Rapeseed meal (RSM), a byproduct from rapeseed oil production, is not only a suitable protein source for swine feed but also a potential energy source. RSM contains a high amount of cell wall polysaccharides, even higher when compared to soybean meal commonly used in the feed industry. A drawback of RSM is that the complex cell wall polysaccharides cannot be utilized by endogenous enzymes from monogastric animals, and also can only be partly fermented by the microbial community in the gastrointestinal tract (GIT). Therefore, enzymatic and chemical treatment on RSM were performed to improve the recalcitrant fibre degradability of RSM.

Chapter 1 reviews the carbohydrate composition in RSM, and polysaccharide degradation in RSM by microbes. The possible processing methods to increase use of recalcitrant fibres are described as well. In addition, the literature was reviewed on microbiota composition in pigs. At the end, different *in vitro* fermentation models were introduced in the chapter, including batch fermentation and dynamic fermentation models (TIM-2, SHIME and SIMGI).

Chapter 2 describes the development of the Swine Large Intestinal *in vitro* Model (SLIM), which was developed based on the human, computer-controlled, dynamic TNO *in vitro* model of the colon, nick-named TIM-2. First, physiological parameters were modified from humans to pigs. Briefly, the temperature was changed from 37 °C to 39 °C, and the pH changed from 5.8 to 5.9. Moreover, the simulated ileal efflux medium (SIEM) used to feed the microbiota in the model was modified from humans to pigs (SIEMP).

In **Chapter 3**, a shot of 5 g (modified) RSM was directly fed to the standardized swine gut microbiota, and the effect of processing methods on the RSM utilization and microbiota composition was studied in SLIM. The present study clearly demonstrated that both enzymatic and chemical pretreatment on RSM shifted its cell wall polysaccharide structure, subsequently altering microbial community composition and functional profile compared to untreated RSM, and eventually increased fibre degradability as evaluated by SCFA production. Furthermore, glycome profiling showed that the abundance of cell wall polysaccharides were dynamically changed during fermentation, and did not continuously decrease during the fermentation period as one might expect.

Continuous fermentation of the modified RSM for 48 h in SLIM was described in **Chapter 4**. The current study demonstrated that microbiota composition was significantly affected by both RSM (processed or not) and adaptation time. Unweighted UniFrac showed that microbial community composition was significantly separated between processed RSM and CON, and CELL and ALK in general changed the microbiota composition more than PECT1 and PECT2 did. Carbohydrate metabolism related microbial functions were predicted to significantly increase after the fibre fermentation period. However, degradability of the processed RSM was not improved compared to CON during the fibre fermentation period, as assessed by SCFA production, which indicated that a relative long adaptation period is needed after a diet change to RSM for swine microbiota.

In **Chapter 5**, the swine gut microbiota was adapted to (modified) RSM for 48 h first, which was followed by a shot of 5 g (modified) RSM as evaluation period. Afterwards, the degradability of (modified) RSM was validated *in vivo* (ileal cannulated pigs) by the mobile nylon bag technique. CELL and ALK feeding considerably changed the swine microbiota structure and functional profile compared to CON, which did not occur with PECT1 and PECT2. The hypothesis is entertained that this results from the different cell wall architecture of RSM once processed by this carbohydrase or alkaline treatment. The increase in relative abundance of pathways involved in carbohydrate fermentation in the microbiota fed CELL- or ALK-treated RSM can be considered as a positive effect of these treatments in fibre

utilization and SCFA production. Moreover, it was validated in pigs that CELL and ALK feeding improved the overall degradation of RSM by the mobile nylon bag technique. With all the data collectively considered, we speculate that carbohydrase enzyme, i.e. CELL, improve fibre degradation of RSM during the fermentation by changing the microbial community structure and enzymatic activity and subsequently shifting the microbiota metagenomic functional profile.

Chapter 6 describes a preliminary novel high-throughput technique to screen polysaccharide structures. The results showed high reproducibility and specificity in recognizing polysaccharides by the glycoprofiling technique, making use of fluorescently labeled carbohydrate binding modules (CBMs), which are fibre recognition domains of amongst others carbohydrases

Samenvatting

Koolzaadmeel (RSM), een bijproduct van de productie van koolzaadolie, is niet alleen een geschikte eiwitbron voor varkensvoer, maar ook een potentiële energiebron. RSM bevat een hoog gehalte aan celwandpolysacchariden, zelfs nog hoger in vergelijking met sojameel dat veel wordt gebruikt in de diervoederindustrie. Een nadeel van RSM is dat de complexe celwandpolysacchariden niet kunnen worden gebruikt door endogene enzymen van monogastrische dieren, en ook slechts gedeeltelijk kunnen worden gefermenteerd door de microbiële gemeenschap in het maagdarmkanaal. Daarom werd een enzymatische en chemische behandeling op RSM uitgevoerd om de weerbarstige vezelafbraak te verbeteren.

Hoofdstuk 1 geeft een overzicht van de koolhydraatsamenstelling in RSM en de afbraak van polysacchariden in RSM door microben. De mogelijke verwerkingsmethode om het gebruik van weerbarstige vezels te vergroten, wordt ook beschreven. Daarnaast werd ook de literatuur samengevat met betrekking tot de samenstelling van de microbiota bij varkens. Uiteindelijk zijn in het hoofdstuk verschillende *in vitro* fermentatiemodellen geïntroduceerd, waaronder batch fermentatie en dynamische fermentatie modellen (TIM-2, SHIME en SIMGI).

Hoofdstuk 2 beschrijft de ontwikkeling van het Swine Large Intestinal *in vitro* Model (SLIM), dat is ontwikkeld op basis van het menselijke, computergestuurde, dynamische TNO *in vitro* model van het colon, met de bijnaam TIM-2. Ten eerste werden fysiologische parameters gewijzigd van mens naar varken. In het kort werd de temperatuur veranderd van 37 ° C naar 39 ° C, en de pH van 5,8 naar 5,9. Tevens werd het gesimuleerde ileale effluxmedium (SIEM), dat wordt gebruikt om de microbiota in het model te voeden, gewijzigd van mens tot varken (SIEMP).

In **Hoofdstuk 3** werd een shot van 5 g (gemodificeerde) RSM direct aan de gestandaardiseerde darmmicrobiota toegevoerd, en het effect van verwerkingsmethoden op het gebruik van RSM en de samenstelling van de microbiota werd bestudeerd in SLIM. De huidige studie toonde duidelijk aan dat zowel enzymatische als chemische voorbehandeling op RSM de polysaccharidestructuur van de celwand verschoof, waardoor de microbiële gemeenschapssamenstelling en het functionele profiel veranderden in vergelijking met onbehandeld RSM, en uiteindelijk de afbreekbaarheid van vezels verhoogde, zoals beoordeeld door korte-keten vetzuur-productie. Bovendien toonde glycome-profilering aan dat de samenstelling van de celwandpolysacchariden tijdens de fermentatie dynamisch veranderde en niet continu afnam tijdens de fermentatieperiode, zoals men zou verwachten. Continue fermentatie van de gemodificeerde RSM gedurende 48 uur in SLIM is beschreven in **Hoofdstuk 4**. De huidige studie toonde aan dat de samenstelling van microbiota significant werd beïnvloed door zowel RSM (al dan niet geprocesseerd) als fermentatietijd. Ongewogen UniFrac toonde aan dat de samenstelling van microbiële gemeenschappen significant gescheiden was tussen verwerkte RSM en CON, en CELL en ALK veranderden in het algemeen de samenstelling van de microbiota meer dan PECT1 en PECT2. Aan koolhydraatmetabolisme gerelateerde microbiële functies waren significant verhoogd na de vezelfermentatieperiode. De afbreekbaarheid van het verwerkte RSM was echter niet verbeterd in vergelijking met CON tijdens de vezelfermentatieperiode, zoals beoordeeld door korte-keten vetzuur-productie, wat erop wees dat een relatief lange aanpassingsperiode nodig is voor microbiota van varkens na een dieetwijziging naar RSM.

In **Hoofdstuk 5** werd de microbiota van de darm van de varkens eerst gedurende 48 uur aangepast aan (gemodificeerd) RSM, gevolgd door een inname van 5 g (gemodificeerd) RSM als evaluatieperiode. Daarna werd de afbreekbaarheid van (gemodificeerd) RSM gevalideerd in een *in vivo* studie (gecanuleerde varkens) door middel van een mobiele nylon zaktechniek. CELL- en ALK-voeding veranderden de microbiota-structuur en het functionele profiel van

varkens aanzienlijk in vergelijking met CON, wat niet voorkwam bij PECT1 en PECT2. De hypothese wordt verondersteld dat dit het gevolg is van de verschillende celwandarchitectuur van RSM, eenmaal verwerkt door deze carbohydrase of alkaline behandeling. De toename van de relatieve overvloed aan microbiële routes die gepaard gaan met koolhydraatfermentatie in CELL of ALK kan worden beschouwd als een positief effect van deze behandelingen bij vezelgebruik en korte-keten vetzuur-productie. Bovendien werd bij varkens gevalideerd dat CELL- en ALK-behandeling de algehele afbraak van RSM verbeterde met behulp van de mobiele nylon zaktechniek. Alle gegevens gezamenlijk beschouwd, speculeren we dat het carbohydrase-enzym, d.w.z. CELL, de afbraak van vezels van RSM tijdens de fermentatie verbetert door de microbiële gemeenschapsstructuur en enzymatische activiteit te veranderen en vervolgens het metagenomische functionele profiel van de microbiota te veranderen.

Hoofdstuk 6 beschrijft een niet volledige nieuwe high-throughput techniek om polysaccharidestructuren te screenen. De resultaten toonden een hoge reproduceerbaarheid en specificiteit aan bij het herkennen van polysacchariden door de glycoprofileringstechniek, gebruikmakend van fluorescent gelabelde koolhydraatbindende modules (CBM's), wat vezelherkenningsdomeinen van onder andere koolhydraat hydrolyserende enzymen zijn.

总结

菜籽粕--生产菜籽油的副产品，不仅是合适的猪的蛋白质饲料原料，而且还是潜在的能源饲料原料。菜籽粕含有大量的细胞壁多糖，与饲料企业中通常使用的豆粕相比，非淀粉多糖含量甚至更高。使用菜籽粕作为饲料原料的一个缺点是复杂的细胞壁多糖不能被单胃动物的内源酶所利用，也只能被肠道中的微生物群落部分发酵。因此，本研究对菜籽粕进行了酶和化学处理，以改善菜籽粕中纤维的降解性。

第1章回顾了菜籽粕中的碳水化合物的组成以及微生物对菜籽粕中多糖的降解。还描述了提高纤维降解率的可能的加工方法。另外，文献回顾了猪的微生物菌落组成。

最后，本章介绍了不同的体外发酵模型（TIM-2，SHIME 和 SIMGI）。

第2章介绍了猪大肠体外模型（SLIM）的开发，该模型是基于人的，全电脑控制的，结肠体外模型（昵称为TIM-2）开发的。首先，将人的生理参数改为猪的生理参数。即，温度从37°C改变为39°C，并且pH从5.8改变为5.9。此外，微生物的培养基已从人的（SIEM）开发为猪的（SIEMP）。

在第3章中，将5克处理过的菜籽粕添加到标准化的猪肠道菌群中，并在SLIM中研究了加工方法对菜籽粕的利用率和菌群组成的影响。本研究清楚地表明，对菜籽粕的酶制剂和化学预处理均改变了其细胞壁多糖结构，与未处理的菜籽粕相比，其改变了微生物群落组成和功能特性，最终提高了纤维的降解率（通过短链脂肪酸（SCFA）的产量评估）。此外，多糖组成分析表明，发酵过程中细胞壁多糖的含量是动态变化的，并且在发酵过程中并没有如人们所预期的那样持续减少。

加工的菜籽粕在SLIM中连续发酵48小时的研究在第4章中进行了描述。当前研究表明，微生物群组成受已加工或未加工的菜籽粕和发酵时间的影响很大。未加权的UniFrac分析表明，经过处理的菜籽粕组和对照组之间的微生物群落组成差异显著，并且CELL和ALK比PECT1和PECT2更能改变微生物群落组成。纤维发酵后，与碳水化合物代谢相关的微生物功能显著增加。但是，通过SCFA产量评估，在纤维发酵期间，与对照组相比，加工后的菜籽粕的降解率并未得到改善，这表明，饲料原料改为菜籽粕后，猪的微生物菌群需要相对较长的适应期。

在第5章中，猪肠道菌群首先适应加工后的菜籽粕48小时，然后添加5克该菜籽粕作为试验期。然后，通过猪的瘘管技术，验证了加工处理确实提高了菜籽粕的降解性。与对照组相比，CELL和ALK喂养大大改变了猪的微生物菌群结构和功能，而PECT1和PECT2则没有。因此我们提出了这样的假设：这是由于通过该酶制剂或碱处理改变了菜籽粕的细胞壁结构所致。与碳水化合物利用相关的微生物丰度的增加对纤维的利用和SCFA的产生有积极作用。此外，通过移动尼龙袋技术证实了在猪饲料中使用CELL和ALK可改善菜籽粕的降解率。综合考虑所有数据，我们推测，通过改变微生物群落结构和酶活性并随后改变微生物菌落的宏基因组功能谱，纤维降解酶制剂（即CELL）可提高菜籽粕中的纤维降解率。

第6章介绍了分析多糖结构的高通量技术。结果显示，通过使用荧光标记的碳水化合物结合模块（carbohydrate binding modules），该技术在糖基分析中具有很高的重现性和特异性。

Impact

It is complicated and costly to investigate the effect of feed fermentation by the gut microbiota in pigs *in vivo*, due to the interaction with other feed-components (such as other fibers) in the swine feed, the ethical considerations (such as using pathogens, toxic compounds, and animal experiments itself) and other uncontrollable factors (such as infectious disease or death during the experiment), which are important for reproducibility of results. *In vitro* fermentative models are considered excellent alternatives to study the effect on the composition and/or activity of the gut microbiota without ethical constraints. They allow the reasonable high throughput screening of a large number of substances such as dietary ingredients and components thereof such as enzyme-, chemical- or physically-treated recalcitrant fibres. In this thesis, a Swine Large Intestinal *in vitro* Model (SLIM) was developed, which was developed based on the human, computer-controlled, dynamic TNO *in vitro* model of the colon, nick-named TIM-2. Therefore, the new developed SLIM can be used by researchers in both universities and feed-related companies to study the scientific questions which cannot be answered *in vivo* as mentioned previously. In this thesis, the SLIM system was used to study the (increased) fermentation of (enzyme- or chemically-processed) recalcitrant fibres in rapeseed meal (RSM), with the aim to increase energy extraction from the diet, through liberation of short-chain fatty acids (SCFA) produced by the pig gut microbiota.

The European Union (EU) is the second-largest importer of raw material for feed (especially protein-rich feed ingredients) in the world, after China. Therefore, in order to have a more sustainable supply of responsible protein-rich ingredients, the European livestock sector needs an alternative local protein feed ingredient to fill the “protein gap”. Rapeseed meal (RSM), a byproduct from rapeseed oil production, is not only a suitable protein source for animal feed but also a potential energy source. RSM contains a high amount of cell wall polysaccharides, even higher when compared to soybean meal commonly used in the feed industry. However, the high amount of pectins in RSM can also reduce the absorption of other nutrients in the gut. Therefore, the aim of the project was to improve utilization of recalcitrant fibre of by-product of rapeseed oil in pigs. Enzymatic and chemical treatment on the RSM were performed in the project to improve the digestibility and fermentability of these by-product in pigs.

The current thesis provides the first detailed analysis of changes in the swine intestinal microbiota due to RSM processed by enzymatic and chemical treatment using the newly developed SLIM system. The present studies clearly demonstrated that both enzymatic (cellulase or two different pectinases) and chemical (6 N sodium hydroxide; alkaline) pretreatment on RSM shifted its cell wall polysaccharide structure, subsequently altering microbial community composition and functional profile compared to untreated RSM, and eventually increased fibre degradability as evaluated by SCFA production in SLIM. Moreover, it was validated in pigs by the mobile nylon bag technique that cellulase and alkaline treatment on RSM improved the overall degradation of RSM. Our findings that the specific treatments increased fibre degradation in RSM could help to guide feed additive strategies to improve efficiency and productivity in swine industry. The current study gave insight into how feed enzymes may modulate microbial status, which provides good opportunity to develop novel carbohydrase, particularly in swine feed. In particular, the genomes of microorganisms that were stimulated by the processed RSM can be mined for novel carbohydrases, that may be used as novel enzymes to pretreat RSM to increase the use of its recalcitrant fibres by the pig gut microbiota.

In order to develop new methods to improve the degradability of plant cell wall

polysaccharides, a better understanding of the cell wall polysaccharide composition and structure and their interactions are essential. In the thesis, a high-throughput technique was developed to screen composition and structure of plant cell wall polysaccharides. The technique (glycome profiling of plant cell wall polysaccharides, or glycoprofilng) was developed with the use of carbohydrate binding modules (CBMs) with unique specificities for plant cell wall polysaccharides, linked to a green fluorescent protein (GFP) as reporter. This toolbox may become essential in the study of the dynamics of breakdown of dietary fibres by the (pig) gut microbiota.

Researchers from both academia and industry can access our results from scientific journals. The result of SLIM model is published in *Beneficial Microbes*, the results of RSM degradation in pigs are published in *Journal of Agricultural and Food Chemistry* and *Frontiers in Microbiology*, and the results of the glycoprofilng technique are prepared for publication. The results call for additional research into recalcitrant fibre fermentation by the (pig) gut microbiota, e.g., by combining multiple enzymes for pretreatment. Both tools (SLIM and glycoprofilng) in our view are pivotal for studying the increased use of feed components by the gut microbiota, leading to increased energy extraction for the host. The use of the tools can be extended to other fibre-sources and other hosts (beyond pigs).

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Thank you!
Dank ye wel!
谢谢！

Cheng

About the author

Curriculum Vitae

Cheng Long was born on the 19th of July 1990 in Pingxiang, China. In 2009, he started his bachelor in Business Administration at Jiangxi Agricultural University, after one semester, and he decided to study Animal Science. In 2013, he completed his bachelor in Animal Science. From here, he went on further, to pursue his master in Animal Nutrition and Feed Science at Chinese Academy of Agricultural Sciences where he worked on

improving egg flavor by inspecting cecal bacteria and trimethylamine concentration when laying hens were administrated rapeseed meal based feed. In September 2016, he started his PhD at Center for Healthy Eating & Food Innovation, Maastricht University. His PhD project was “Improving intestinal degradation of recalcitrant fiber in pigs”, where he was working on illuminating the characterization of the inaccessible cell wall architecture and the effect of modified fibres on swine gut microbiota. During this project, he also closely cooperated with Group of Animal Nutrition and Laboratory of Food Chemistry at Wageningen University. The outcome of his research during his PhD is presented in this thesis.

List of Publications

Long C, de Vries S, Venema K. Polysaccharides source alters molecular ecological network interaction, functional profile, and short-chain fatty acid production in a porcine gut microbiota. *Beneficial Microbes*. 2020;11 (6), 591-610. DOI: 10.3920/BM2020.0006.

Long C, de Vries S, Venema K. Differently Pre-treated Rapeseed Meals Affect in vitro Swine Gut Microbiota Composition. *Frontiers in Microbiology*. 2020;11(2125). DOI: 10.3389/fmicb.2020.570985.

Long C, Rösch C, de Vries Sonja, Schols HA, Venema K. Cellulase and alkaline treatment rather than pectinase improves intestinal microbial degradation of recalcitrant fibres of rapeseed meal in pigs. *Journal of Agricultural and Food Chemistry*. 2020: 68(39) 11011–11025. DOI: 10.1021/acs.jafc.0c03618

Long, C, Venema K. Pretreatment of rapeseed meal increases its recalcitrant fibre fermentation and alters the microbial community in an in vitro model of swine large intestine. *Frontiers in Microbiology*. 2020, DOI: 10.3389/fmicb.2020.588264.

Long, C, Venema K. Chemical and nutritional characteristics, and microbial degradation of rapeseed meal recalcitrant carbohydrates. Submitted for publication.

Long, C, Venema K. High-throughput screening of structures of plant cell wall polysaccharides using a novel method. Prepared for publication.

Other work:

Long, C., Wang, J., Zhang, H.J., Wu, S.G. and Qi, G.H., 2017. Effects of dietary rapeseed meal supplementation on cecal microbiota in laying hens with different flavin-containing monooxygenase 3 genotypes. *Poultry science*, 96(6), pp.1748-1758.

Long C, Chen L, Wang J, Wu SG, Zhang HJ, Yue HY, Qi GH. Effects of Dietary Canola Meal Level on Performance, Egg Quality, Trimethylamine Content in Egg Yolk of Flavin-Containing Monooxygenases 3 Genotype Laying Hens[J]. *Chinese Journal of Animal Nutrition*, 2016, 28(6): 1687-1695.

Long C, Wang J, Wu SG, Zhang HJ, Yue HY, Qi GH. Influence of Feed Factors on Egg Flavor and the Measures for Flavor Improvement [J]. *Chinese Journal of Animal Nutrition*, 2015, 27(2):352-358.

Wang, J., **Long, C.**, Zhang, H., Zhang, Y., Wang, H., Yue, H., Wang, X., Wu, S. and Qi, G., 2016. Genetic variant in Flavin-containing monooxygenase 3 alters lipid metabolism in laying hens in a diet-specific manner. *International journal of biological sciences*, 12(11), p.1382.

Wang, J., Zhang, H.J., Xu, L., **Long, C.**, Samuel, K.G., Yue, H.Y., Sun, L.L., Wu, S.G. and Qi, G.H., 2016. Dietary supplementation of pyrroloquinoline quinone disodium protects

against oxidative stress and liver damage in laying hens fed an oxidized sunflower oil-added diet. *animal*, 10(7), pp.1129-1136.

Wang, J., Zhang, H.J., Samuel, K.G., **Long, C.**, Wu, S.G., Yue, H.Y., Sun, L.L. and Qi, G.H., 2015. Effects of dietary pyrroloquinoline quinone disodium on growth, carcass characteristics, redox status, and mitochondria metabolism in broilers. *Poultry Science*, 94(2), pp.215-225.

Overview of completed training activities

Discipline specific courses

Courses

The Intestinal Microbiome and Diet in Human and Animal Health, Wageningen, The Netherlands, 2017

15th European Training Course on Carbohydrates, Wageningen, The Netherlands, 2018

Gut health in pigs and poultry, Wageningen, The Netherlands, 2019

Conferences

The 6th Beneficial Microbes Conference, Amsterdam, The Netherlands, 2017

The 19th Gutday, Venlo, The Netherlands, 2017

14th International Symposium Digestive Physiology of Pigs, Brisbane, Australia, 2018

The 7th Beneficial Microbes Conference, Amsterdam, The Netherlands, 2018

The 20th Gutday, Middleburg, The Netherlands, 2018

General courses

Effective communication Intermediate, Maastricht, The Netherlands, 2017

Biosafety in the laboratory, Maastricht, The Netherlands, 2018