The role of soluble and insoluble fibers during fermentation of Chicory root pulp

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Abstract

This thesis was aimed at understanding the *in vitro* fermentability of soluble and insoluble fibers in chicory root pulp (CRP). First, CRP and ensiled chicory root pulp (ECRP) were characterized for cell wall polysaccharides (CWPs). Both CRP and ECRP were rich in CWPs (56-58 w/w (%)) and had rather similar sugar compositions. The CWPs consist of 62 % pectin, 11% hemicellulose and 27% cellulose. Pectin and xyloglucan were acetylated and the rhamnogalacturonan-I segments of pectin were branched mostly with arabinan. Compared to CRP, ECRP has four times more soluble pectin.

In vitro fermentability in a batch model for 24 h using human faecal inoculum, showed that fibers in both CRP (51% carbohydrate utilisation) and ECRP (59% carbohydrate utilisation) were fermentable, especially pectin (80-87%). The increased levels of soluble pectin (arabinan, homogalacturonan and galactan) and the hypothesized open cell wall structure in ECRP contributed to a quicker fermentation and a higher level of carbohydrate utilization compared to CRP. In contrast to batch fermentation, fermentation in the dynamic TNO *In vitro* model of the colon (TIM-2) was rapid (57% carbohydrate utilisation in 2 h). ECRP carbohydrates (85%) were less fermented in 24 h compared to CRP carbohydrates (92%) due to lower utilisation of ECRP insoluble fibers than CRP insoluble fibers. It was hypothesized that soluble fibers that are readily fermentable and dominantly present in ECRP, programmed the microbiota in TIM-2 to fully adapt to these soluble fibers. After their utilization, the microbiota was not able to adapt towards the fermentation of insoluble fibers.

Analysis of enzyme activities during batch fermentation of CRP showed increased levels of arabinofuranosidase, β-galactosidase, endo-arabinanase, endo-galactanase, exo-polygalacturonase, pectin de-esterifying enzymes and endo-polygalacturonase. They synergistically contributed to degrading pectin in CRP from 12 to 24 h of fermentation.

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Chapter 1

General introduction

1.1. Background

The use of fibers from agricultural by-products for food purposes is gaining importance. Most commonly used for food purposes are fibers arising from the cereal processing industry *(1)*. The cereal based by-products are abundant in xylan and β-glucan fibers *(1, 2)*. In contrast, by-products from the fruit and vegetable processing industry, which are dominant in pectin fibers, are very limited in use *(3)*.

Chicory root pulp (CRP) is an agricultural by-product obtained after industrial extraction of inulin from the chicory root. It is rich in pectin fibers, followed by cellulose and hemicellulose *(3)*. The pulp also contains some residual inulin, which has not been completely removed during the industrial extraction process. For economic reasons, the agricultural by-product is used for animal feed. In order to substantiate the use of the pulp as a fiber supplement for human consumption, a multidisciplinary project 'Novel food fibers' was started. Main research areas in this project dealt with determining the chemical and techno-functional characteristics of fibers in CRP, studying *in vitro* and *in vivo* effects of CRP and immune-modulatory effects of defined fibers as present in CRP. This thesis, as a part of the above mentioned project, aimed at understanding the effect of CRP cell wall polysaccharides and their arrangement in the cell wall network on the *in vitro* fermentability of polysaccharides by human colonic bacteria.

1.2. Dietary fibers

Dietary fibers have been claimed to contribute to health benefits, such as adding bulk and softness to stool *(4, 5)*, controlling blood glucose levels *(6)*, lowering blood cholesterol levels *(7)*, providing immuno-modulatory effects *(8)*, providing satiety and facilitating weight reduction *(9, 10)* and reducing the risks of gastrointestinal disorders and colon cancer *(11)*. The term dietary fiber (DF) encompasses plant cell wall polysaccharides (CWPs), such as pectin, hemicellulose and cellulose, which are resistant to digestion in the human small intestine and become available for (partial) fermentation in the colon by the human microbiota *(12)*.

1.3. Plant cell wall polysaccharides

Plant cell walls support cell membranes surrounding plant cells and provide rigidity, strength and shape to plant cells *(13)*. They are built of polysaccharides, structural proteins and lignin *(14, 15)*. The plant cell wall in dicotyledonous plants mainly consists of the middle lamella and the primary cell wall. The middle lamella consists mostly of pectic polysaccharides which glue plant cells together while the primary cell wall is characterized by a higher degree of organization of polysaccharide *(16)* giving the cell wall rigidity and structure (figure 1.1). Normally, the primary cell wall of dicotyledonous plants contains around 35% pectin, 30% cellulose, 30% hemicellulose and 5% protein *(17, 18)*. In contrast, the primary cell wall of monocots are abundant in arabinoxylan and cellulose, while they contain <10% pectin *(19)*.

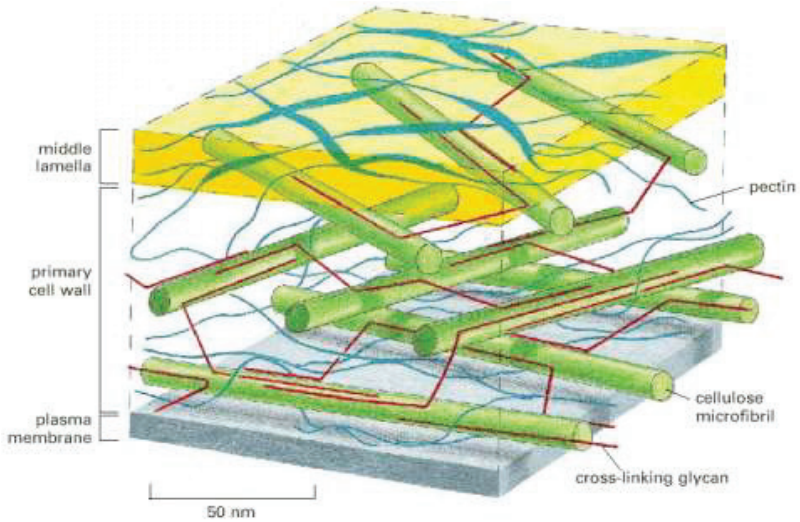

Figure 1-1. Schematical plant cell wall model of dicotyledons as proposed by McCann and Roberts (*20*).

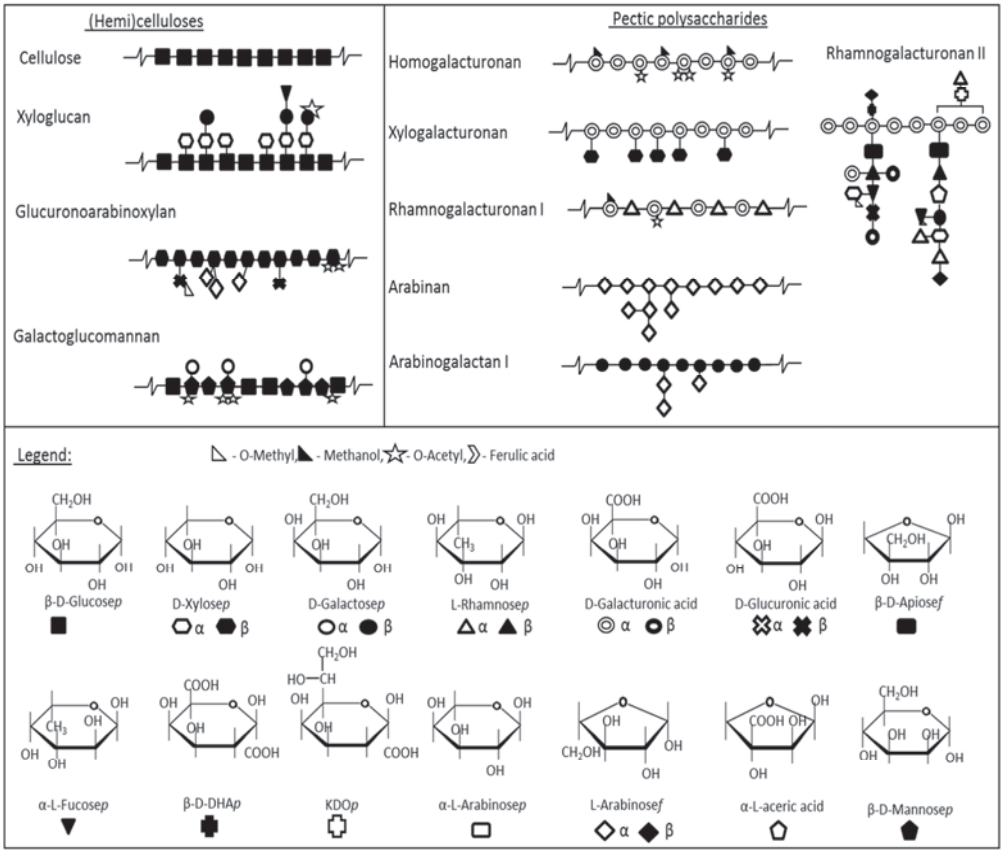

Figure 1-2. Schematic representation of plant polysaccharide structures. DHA: 3-deoxy-D-manno-2-octulosonic acid; KDO: 3-deoxy-D-lyxo-2-heptulosaric acid.

The chemical structure and variations of polysaccharides are described below:

Pectin

Pectin is a heterogenous group of polysaccharides consisting of homogalacturonan (HG), rhamnogalacturonan (RG) I and II, arabinan, galactan, arabinogalactan and xylogalacaturonan (XGA).

HG. Pectin mostly comprises HG which contains α-D-galacturonic acid (GalA) residues linked by 1,4-linkages *(21)*. The GalA residues may be esterified with methanol at C-6 and/or acetylated at O-2 and /or O-3 *(22)* (figure 1.2). The distribution and the extent to which methyl esters and acetyl groups are present vary from source to source.

HG may have different substitutions such as present in: i) *RG II*: This structural element is well conserved and present in low levels. Sugars of 2-O-methyl xylose, 2-O-methyl fucose, apiose, aceric acid, 2-keto-3-deoxy-D-lyxo heptulosaric acid (DHA) and 2-keto-3-deoxy-D-manno octulosonic acid (kdo) form four well defined side chains of a backbone of nine GalA residues to form the RG II *(23)* (figure 1.2). ii) *Xylogalacturonan (XGA)*: HG is substituted with single xylose units *(24)* or iii) *Apiogalacturonan (AGA)*: HG is substituted with apiose residues to form AGA aspresent in duck weed *(25,* 26).

RG I. The RG I consists of alternating α-(1,2)-linked rhamnosyl and α-(1,4)-linked galacturonic acid residues *(27)*. The rhamnosyl residues can be substituted at O-4 by side chains composed of arabinose and/or galactose residues forming *arabinans, galactans and arabinogalactans (AG) I and II (28)* (figure 1.2). *Arabinan* is composed of a backbone of α-L-(1,5)-linked arabinofuranosyl residues, which may be substituted with α-arabinofuranosyl residues at the O-2 and/or O-3 position *(15, 29, 30)* (figure 1.2). The backbone of *AG I* is composed of β-1,4 linked galactose units which are substituted with α-1,5- linked arabinose units at the O-3 position *(15)*. The backbone of *AG II* is composed of β-1,3 linked galactose units which are substituted with galactose units at O-6 which may be further substituted with single arabinose residues *(31)* (figure 1.2).

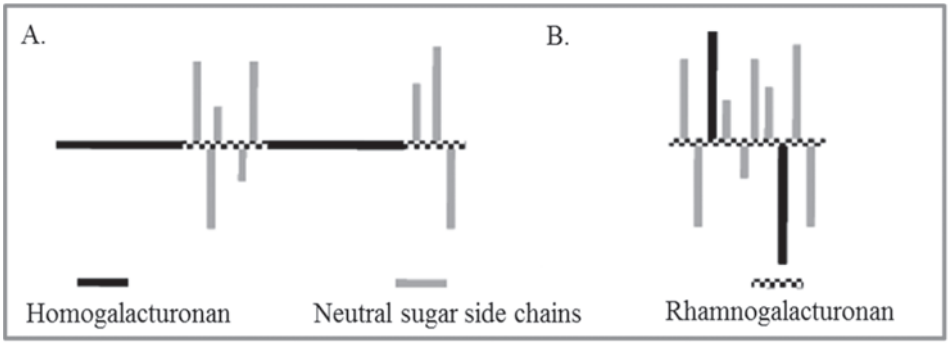

Figure 1-3. Schematic representation of two different models A. the smooth and hairy regions model and B. the rhamnogalacturonan model describing the hypothetical pectin structure, adapted from Vincken et al (*32*).

Arabinan, galactan and arabinogalactan I and II present as neutral sugar side chains of rhamnogalacturonan (RG) constitute the so called *hairy regions of pectin (33)*. Two popular models are used to describe the pectin structure: *The smooth and hairy region model (21)*

and the *RG I backbone model (32)*(figure 1.3). In the first model, the hairy regions are interspersed with smooth regions HG containing 70-100 GalA residues *(21, 34)*. In addition, RG II is believed to be an integral part of HG *(35)*. In the second model, HG is a side chain of RG I as are the neutral sugar side chains *(32)*.

Cellulose

Cellulose is insoluble and provides a framework to plant cells. Cellulose is built of β-1,4 linked glucose units (figure 1.2) which make a fibril. An association of many crystalline densely packed fibrils together with less ordered, amorphous regions of less densely packed cellulose together forms a microfibril. The microfibrils are hydrogen bonded along their length *(14, 36, 37)*.

Hemicellulose

Hemicellulose is a class of many different polysaccharides, such as, xyloglucans, xylans and mannans. Predominant polysaccharides in dicotyledons are *xyloglucans*. These polymers have a β-1,4 linked backbone of glucose residues as in cellulose but which is 75 % of the residues are substituted at O-6 by α-xylose (figure 1.2). Further extensions of these xylose side chains may include arabinosyl, galactosyl or fucosyl units at O-2 position *(38-40)*. In addition, O-acetylation of galactose and/or glucose residues is observed *(39)*. Xyloglucans bind to cellulose microfibrils in the native cell wall by hydrogen bond interactions *(38)*.

Xylans have a β-1,4 linked backbone of xylose residues, which may be unsubstituted as homoxylans or may be substituted with arabinose or glucuronic acid or both to form arabinoxylans, glucuronoxylans or glucuronoarabinoxylans respectively (figure 1.2). They are characteristic of monocots such as, cereals *(26, 41, 42)*. In dicots, such as potato and sugar beet, they are also present, but in low quantities *(42)*.

Mannans have a mannose β-1,4 linked backbone (figure 1.2). The backbone may be substituted with α-1,6-linked galactose residues forming galactomanans. Mannans may also contain glucose units in their backbone forming (galacto)glucomannans *(43)*.

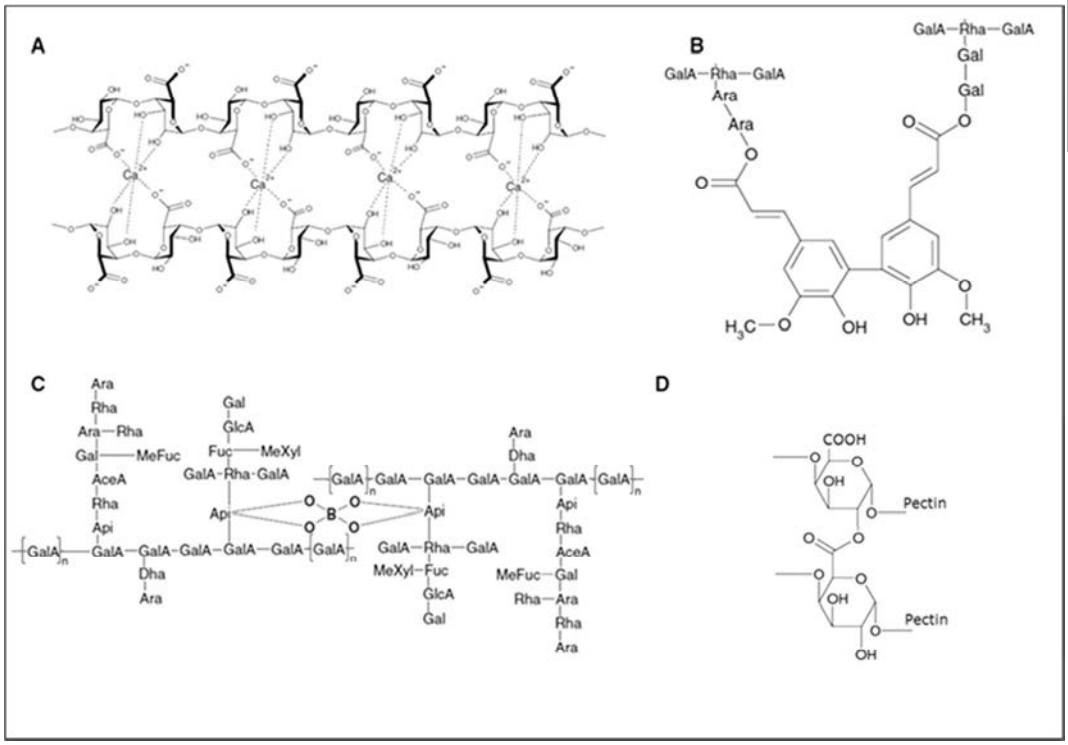

Figure 1-4. Schematic presentation of possible pectin- pectin crosslinks in a cell wall: A. Egg box model of calcium-pectin crosslink *(44)*, B. Ferulic acid crosslink, for example 5-5-diferulic acid esterfied with neutral sugar side chains of pectin *(45)*, C. Rhamnogalacturonan II diester, *(35)*, D. Uronyl ester of pectin with a hydroxyl group of another polysaccharide chain *(46)*.

Cell wall network / architecture

Cellulose microfibrils and xyloglucan are interlinked via hydrogen bonds forming a rigid network *(15, 47)* (figure 1.1). Depending on the proportions of xyloglucan and pectin in the cell wall, pectin may as well interact with cellulose *(48)*. For cell walls containing reasonable levels of xyloglucan as in pea *(49)* and blackberries *(50)*, pectins bind loosely to cellulose and fill the interstices within the cellulose/ xyloglucan network *(48)*. In case of relatively low amounts of xyloglucan, e.g. in sugar beet *(51)*, pectins replace xyloglucan as they bind to cellulose microfibrils *(48, 52)*.

Pectins have also been shown to interact with hemicellulose via its neutral sugar side chains *(47)*. Pectins also interact with proteins via arabino-3,6-galactans *(53)*. Proteins in the cell wall are mostly hydroxyproline rich glycoproteins which include extensins, arabinogalactan

proteins, and lectins *(31)*. Pectins may also be linked with each other via the following crosslinks (see also figure 1.4):

A. *Calcium crosslinking between unesterified GalA residues.* Usually, blocks of more than 10 unesterified GalA residues give rise to pectin molecules sensitive to Ca^{2+} crosslinking *(44)*.
B. *Ferulic acid crosslinking.* The propenoic double bonds of ferulic acid may crosslink pectins *(45)*. The ferulic acids are found in RG I and are attached to the O-2 position of arabinose and to O-6 position of galactose *(45, 54-56)*. Usually found in low concentrations, sugar beet pulp contains 0.7% ferulic acid of cell wall material. Around 22 % of the total ferulates exist as ferulic acid dehydromers in sugar beet pectin *(56)*.
C. *Rhamnogalacturonan (RG) II boron diester crosslinking.* RGII components are crosslinked via a borate diester *(35)*. RG II is present predominantly as a dimer (figure 1.4) in plant cell walls *(57)*.
D. *Uronyl ester crosslinking.* A covalent ester bond is formed between galacturonic acid in pectins and hydroxyl group of neighbouring polysaccharides crosslinking two (pectin) chains *(46, 50)*. However, strong evidence for the presence of such esters is still lacking.

1.4. Enzymes degrading plant cell wall polysaccharides

In general, CWPs can be degraded by two classes of enzymes: i. Glycosidases that hydrolyse single sugars or small oligomers from the ends of oligomers and exo-enzymes that hydrolyse from the ends of polymers and ii. Endo enzymes that cleave linkages within a polymer backbone give rise to a drastic decrease of the molar mass of the polymer. Three types of cell wall degrading enzymes exist: 1. Glycosyl Hydrolases (E.C. 3.2.1) cleave the glycosidic linkage between two sugar units and introduce a water molecule, 2. Polysaccharide Lyases (E.C. 4.2.2) which cleave the glycosidic linkage by introducing a double bond and 3. Carbohydrate Esterases (E.C. 3.1.1) that remove non carbohydrate substituents such as methyl esters, ferulic acid groups and acetyl groups from

carbohydrates. Degradation of each polysaccharide requires a specific set of enzymes as discussed below:

Cellulose degrading enzymes

Cellulose can be degraded by three types of enzymes. i. Endo-glucanases which hydrolyse cellulose randomly producing reducing ends; ii. Exo-glucanases which liberate D-glucose from cellulose or cellodextrin and also liberate cellobiose (cellobiohydrolase) from cellulose in a processive manner and iii. β-glucosidases which form D-glucose from cellobiose *(58)*. Hydrolysis of crystalline cellulose is made efficient by the carbohydrate binding modules (CBM) linked to cellulases. CBMs attach to the substrate and the linker between the CBM and the catalytic site provides sufficient freedom for the catalytic domain to move around the binding module attached to the substrate. This increases the proximity of the catalytic domain to the substrate for hydrolysis *(59)*.

Hemicellulose degrading enzymes

Xyloglucan degrading enzymes. Xyloglucan requires different enzymes for degradation of the backbone and the side chains. Backbone degradation is either by non-specific endo-glucanase or xyloglucan specific endo-glucanase and β-glucosidases *(40, 60)*. Side chains are degraded by α-xylosidases, β-galactosidases and acetyl esterases *(60, 61)*. Xylan degrading enzymes: Xylan backbone can be degraded by endo-xylanases and β-xylosidases whereas arabinose substituents can be removed by arabinofuranosidase *(60)*. Glucuronic acid can be cleaved by glucuronidases (*62*).

Mannan degrading enzymes. The mannan backbone is degraded by endo-mannanase *(60)* while the linkage between glucose residues in the mannan backbone can be hydrolysed by endoglucanase and side chains of mannan are degraded by α-galactosidase.

Pectin degrading enzymes

Different enzymes are required for degrading pectic polysaccharides. These enzymes are shown in figure 1.5.

The HG can be degraded by exo- and endo-polygalacturonase. The action of endopolygalacturonase is hindered by esterified GalA residues, due to which other enzymes, such as pectin methyl esterase and pectin acetyl esterase, are also required to enhance degradation of esterified HG. Besides, esterified HG can also be cleaved by pectin lyase (figure 1.5).

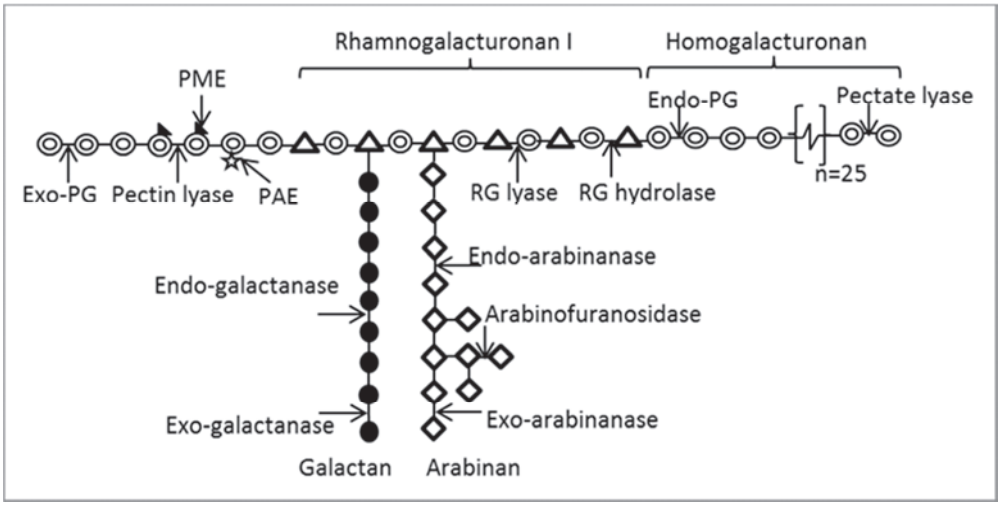

Figure 1-5. Sites of action by pectin degrading enzymes. PG, polygalacturonase; PME, pectin methyl esterase, PAE, pectin acetyl esterase; RG, rhamnogalacturonan *(63)*

RG I degradation is catalysed by RG-hydrolases. The RG galacturonohydrolase liberates galacturonic acid from RG *(64)* while the RG rhamnohydrolase is an exo-enzyme which releases rhamnose from the non-reducing end of RG and RG rhamnogalacturonohydrolase is an endo-enzyme which cleaves between rhamnose and galacturonic acid *(65)*. RG is cleaved by RG lyase (RGL) *(66)*. Esters on RG I are also removed by RG-acetyl esterases. Acetyl groups and side chains attached to Rha hinder the action of RGH and RGL.

Arabinan can be degraded by endoarabinanases, which cleave α-1,5-linkages within a linear backbone (*67*). Exo-arabinanases cleave arabinose from the non-reducing end of the α-1,5 linked backbone (*68*) or arabinotriose from branched arabinan (*69*). Arabinofuranosidases hydrolyse α-arabinofuranosyl residues present as terminal substituents on the α-1,5 linked backbone and on branched arabinan (*70*). Degradation of galactan and the backbone of AG I and AG II requires activities of endo-galactanase and β-galactosidase. 1,4 galactanase cleaves AG I backbone and 1,3,6 galactanase cleaves the backbone of AG II. Arabinose substituents are removed by arabinofuranosidase and arabinopyranosidase.

1.5. Fiber rich agricultural by-products

Agricultural by-products from industrial processing include roots and tubers *(71-73)*, vegetables and fruits *(74, 75)*, and cereals *(76, 77)*.

Table 1-1. Constituent monosaccharide content of agricultural by-products

	w/w %									
Sources	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total	Reference
Chicory root pulp	1	0	7	3	2	4	23	28	69	(*78*)
Sugar beet pulp	1	0	22	2	1	5	23	13	67	(*79*)
Potato pulp	2	0	4	1	1	12	14	11	44	(*80*)
Apple pulp	1	1	8	6	1	6	29	24	76	(*81*)
Rape seed meal	2	0	19	8	6	10	40	15	36	(*82*)
Sunflower cake	0	0	3	6	1	1	13	7	32	(*77*)
Alfafa meal	0	0	3	6	1	2	15	7	33	(*77*)
Soybean meal	0	0	3	2	1	4	7	5	22	(*77*)
Linseed meal	1	0	4	7	0	3	12	4	30	(*77*)
Corn fiber	0	0	12	18	2*	3	14	4	53	(*83*)
Corn cobs	0	0	2	28	1*	1	33	2	67	(*83*)
Corn stover	0	0	3	19	1*	1	30	2	56	(*83*)
Brewers spent grain	0	0	8	15	0	1	16	2	42	(*84*)
Palm cake	0	0	1	3	31	2	8	2	47	(*77*)
Wheat bran pulp	0	0	22	32	0	1	21	2	79	(*77*)
Coconut cake	0	0	1	1	31	3	6	1	42	(*77*)
Rye bran	0	0	8	21	0	1	11	1	42	(*77*)
Barley dehulled	0	0	2	4	0	0	6	0	13	(*77*)
Oats feed meal	0	0	1	2	0	0	5	1	9	(*77*)

Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA), *Values represent Man+Rha.

They have been used for feed purposes for reasons such as, high dietary fiber content, high protein content etc *(76, 77)*. Agricultural by-products rich in polysaccharides are shown in table 1.1. These by-products have been utilised for feed purposes.

As shown, the by-products may differ significantly in the constituent monosaccharide composition. The by-products can be broadly distinguished as pectin rich by-products, such as CRP, sugar beet pulp, potato pulp, apple pulp and rape seed meal and (hemi)cellulose rich by-products. Among (hemi)cellulose rich by-products, mannan is abundant in palm cake (31 w/w%) and coconut cake (31 w/w%) whereas xylans are abundant in corn fiber

(18 w/w%), corn cobs (28 w/w%), corn stover (19 w/w%), brewers spent grain (15 w/w%), wheat bran pulp (32 w/w%), and rye bran (21 w/w%). Differences in pectic polysaccharides can also be seen within pectin rich by-products. The pectin in CRP contains less arabinan (7 w/w %) compared to sugar beet pulp (22 w/w %).

Chicory root pulp

Chicory (*Cichorium intybus L.*) root is used industrially for the extraction of inulin. The pulp obtained is used as animal feed. Pectin in chicory root pulp is highly methyl esterified (68) and acetylated (36)*(78)*. The gelling behaviour of chicory root pectins extracted by a combination of protease and cellulase enzymes has been studied *(85)*. It was found that chicory root pectins gelled with sucrose at an acidic pH, but not as strong as citrus pectins that have a higher GalA content than chicory root pectins. Research on chicory root cell wall fibres has only been started since a few years *(3, 78, 85, 86)*. Hence, not much information is available on the different populations of polysaccharides and their interactions in CRP.

Water holding capacity (WHC) of cell wall materials

Physical attributes of cell wall materials, such as water holding capacity (WHC), have an influence on their functionality such as fermentability or in application of fibers to food. The WHC is in turn determined by the molecular structure of the CWPs and the cell wall architecture *(87)*. Sugar beet pulp has a WHC (5.5 mL/g) *(88)*. Treatments like autoclaving or grinding of sugar beet pulp reduced the WHC of sugar beet pulp *(89)*. The WHC could also be modulated by different means such as enzymatic treatment of the plant cell wall or processing of the plant cell wall.

Agricultural by-products containing plant cell wall polysaccharides may be fermented to different extents in the colon. The fermentation of such plant cell wall based dietary fibers is elaborated in the following section.

1.6. Fermentation of dietary fibers

Fermentation of dietary fibers in the colon is determined by the colonic bacteria and the type of fibers entering the colon. The colon is densely populated with microbiota (mostly anaerobic) accumulating to approximately 10^{12} bacteria per g dry weight of colonic contents

(90). The type of bacteria present in the colon determines the health of the colon. Figure 1.6 *(91)* shows the dominant populations of bacteria in the colon.

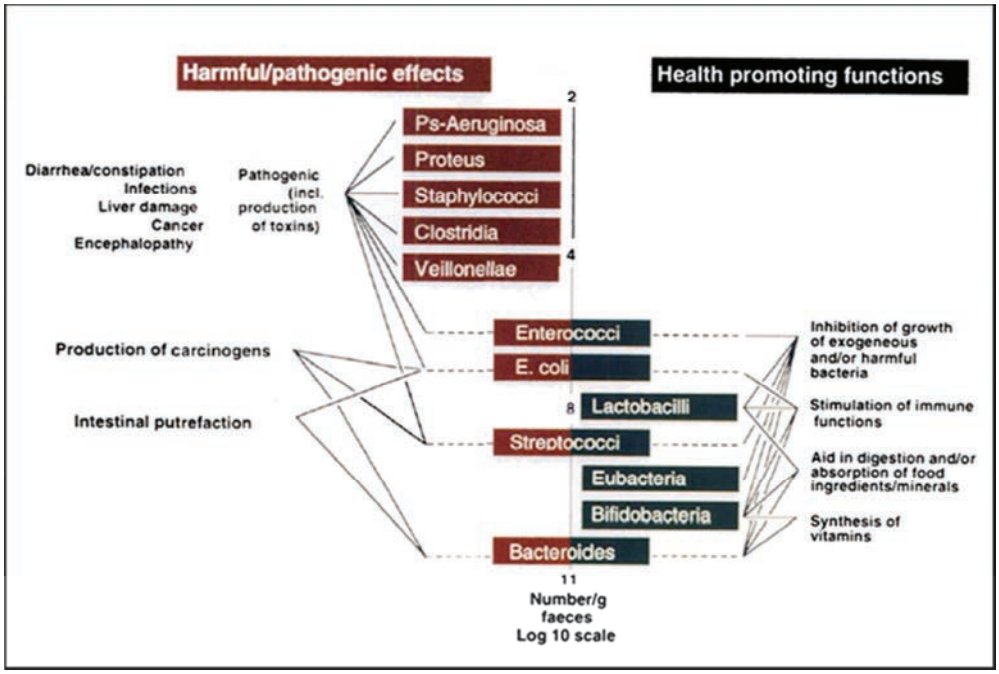

Figure 1-6. Schematic presentation of composition and health effects of the predominant human faecal bacteria *(91)*.

Most abundant bacteria belong to the genus *Bacteroides*, which are partly health promoting and partly harmful. The health promoting bacteria include *Bifidobacteria, Eubacteria and Lactobacilli*. *Streptococci, E.coli,* and *Enterococci* are partly beneficial and partly pathogenic, whereas *Veillonellae, Clostridia, Staphylococci, Proteus, Pseudomonas aeruginosa* are pathogenic when present in the colon *(91)*.

The proportions of species in the colon could change depending on the type of fiber consumed. Examples of bacteria that degrade different types of polysaccharides are shown in table 1.2. Most bacteria that degrade cell wall polysaccharides fall in two genera, *Bifidobacteria* and *Bacteroides (92)*. *Bacteroides ovatus, Bacteroides thetaiotaomicron and Bacteroides uniformis* were able to ferment an array of polysaccharides *(92)*. Fermentation of oligosaccharides using human faecal inoculum showed that rhamnogalacturono-

oligosaccharides were only fermentable by *Bacteroides spp.(93).* Highly branched xylans from wheat flour and sorghum were also fermentable by *Bacteroides spp. (93).*

Table 1-2. Polysaccharides that are fermentable by intestinal bacteria *(92, 94, 95)*

Polysaccharide	Bacteria able to utilize polysaccharides
Pectin (<70% galacturonic acid)	*B. thetaiotaomicron, B. ovatus, Bacteroides "3452 A", B. fragilis* subspecies a, *B. vulgatus, Eubacterium eligens*
Xylan (linear)	*B. ovatus, B. eggerthii, B. fragilis* subspecies a, *B. vulgatus, Bifidobacterium adolescentis, Bifidobacterium infantis*
Galactomannans Guar gum (mannose:galactose 1.8) locust bean gum (mannose:galactose 3.5)	*B. ovatus, B. uniformis, Ruminococcus albus*
Arabinogalactan	*B. thetaiotaomicron, B. ovatus, B. '3452 A ', B. uniformis, B. vulgatus, B. "T4-1 Bıfldobacterium longum*
Cellulose	*Bacteroides sp.*

Enzymes produced by the colonic microbiota

B. thetaiotaomicron known to be highly abundant among *Bacteroides sp. (96)* has been estimated to include 236 glycoside hydrolase genes, 15 polysaccharide lyase genes, 20 carbohydrate esterase genes and 16 carbohydrate binding modules *(97-99).* Expression of enzymes by these genes during the course of fermentation is up-regulated based on the polysaccharides present during fermentation *(97).*

The enzymes active in degrading polysaccharides during fermentation may be extracellular or bound to the bacterial cell wall. Degradation of soluble viscous polysaccharides has been found to involve extracellular enzymes whereas degradation of insoluble polysaccharides by *Bacteroides* has been found to involve cell wall bound enzymes *(92).* Degradation of an insoluble cell wall material first requires the adhesion of fermenting bacteria to the cell wall polysaccharide *(100).* The mechanism of adhesion, however, is poorly understood. It might

involve substrate binding modules from enzymes and structural proteins and carbohydrate moieties on glycoproteins *(98)*.

Fermentation metabolites

Fermentation of DFs in the colon produces short chain fatty acid (SCFAs), such as acetate, butyrate and propionate, and organic acids, such as succinate and lactate, as well as gases such as hydrogen and methane *(91)*. The organic acids are usually intermediates that are converted to SCFAs. These are further metabolized (consumed) by the host as an energy source *(101)* and also contribute to health benefits. Propionate is gluconeogenetic (synthesis of glucose) and inhibits the synthesis of cholesterol from acetate, thus at least controlling the levels of serum cholesterol *(102)*. Butyrate has beneficial effects on inflammatory responses *(103)* and on the cell cycle. It promotes cellular differentiation, has anti-neoplastic properties and has effects on apoptosis *(104, 105)*.

Cross feeding of polysaccharides by bacteria and competition also exist among bacterial populations *(106)*. For example, oligofructose and inulin are known to cause a bifidogenic shift (increase in *Bifidobacteria*) in the microbial composition of the colon *(107)*. The subsequent increase in butyrate is not caused by *Bifidobacteria* but by other colonic bacteria, thereby suggesting crossfeeding between *Bifidobacteria* and butyrate producing bacteria *(106)*. Crossfeeding has also been demonstrated between resistant starch degrading *Bifidobacteria* and lactate-converting, butyrate producing colon bacteria *(108, 109)*.

Models used for studying colonic fermentation

Fermentation of DF can be studied in different types of models generally classified as *in vitro* or *in vivo* models. Compared to *in vivo* fermentation models, *in vitro* fermentation models can be used without ethical constraints and they enable analysis of time dependant degradation of fiber in the colon. In addition, *in vitro* fermentation experiments are more simple to perform than *in vivo* fermentation experiments and there is no interference of other food components in the investigation of the effect of fibers themselves.

In vitro fermentation of fibers can be studied in numerous types of models. The simplest of the models commonly used is a closed system model represented by sealed bottles containing suspensions of faecal microbiota, fiber substrates and being maintained under anaerobic conditions *(110)*. Such batch fermentations have been performed on fibers, such

as guar gum, alginate, retrograded starch, glucomannan, cellulose, β-glucans, inulin, oligofructose, high methyl esterified citrus pectin, soy pectin and xanthum gum *(111)*, beet pulp *(112, 113)* and citrus pulp *(113)* using human faecal inoculum.

In contrast to batch models, dynamic *in vitro* gut fermentation models are single or multiple chemostats inoculated with fecal microbiota and operated under physiological temperature, pH and anaerobic conditions *(110, 114-117)*. Single chemostats, such as the TIM-2 (TNO *In Vitro* Model)(*116*) are used to mimic the proximal colon while multiple chemostats are used to mimic the proximal, central and descending parts of the colon such as the three stage culture model *(115)*. The TIM-2 has been combined with TIM-1 (small intestinal model) *(118)* resulting in a digestive system to study drug delivery and advanced nutritional studies *(119-121)*.

Although fermentation in a batch model is simple, inexpensive and high throughput, the fermentation requires a strong buffer to control the pH due to the accumulation of main fermentation metabolites SCFA. In contrast, in continuous gut fermentation models, the fermentation metabolites are not accumulated but dialysed out. Water is absorbed and fermentation metabolites are removed via a hollow fiber membrane in TIM-2 (*116*). In addition, due to peristaltic mixing of components, such models are closer to simulating the human colon than batch models. TIM-2 also has the capacity to handle high microbial densities as found *in vivo* and high substrate concentrations compared to the batch fermentation (*116*).

Effect of polysaccharide arrangement on fermentability

Although dietary fibers (e.g. by-products) contain pectin, hemicellulose and cellulose, differences in the structural features of individual polysaccharides and the molecular arrangement of these polysaccharides and can contribute to different levels of fermentation *(112)*. Differences in fermentability have also been shown for cell wall materials having the same constituent monosaccharide composition but modified cell wall networks by processing. *In vitro* fermentation of beet pulp and autoclaved sugar beet pulp with a different polysaccharide network than beet pulp showed that removal of pectic polysaccharides by autoclaving increased the degradability of cellulose. This suggested that accessibility of a polysaccharide within the network towards fermentation enzymes depends

on the arrangement of polysaccharides within a network *(89)*. This also suggested that solubilisation of fibers from a network or increase in soluble fiber levels makes the remaining polysaccharides in the insoluble network more accessible towards enzymes *(112)*.

Delayed fermentability of a cell wall material has positive effects, such as, preventing proteolytic fermentation in the colon as the cell wall material passes through the colon. Proteolytic fermentation is undesired because it leads to the production of ammonia amongst other metabolites, and thereby leads to a pH that is near neutral to allow growth of pathogens and increase vulnerability of the distal colon to chronic gut disorders *(122-126)*.

Physical properties of cell wall materials such as, WHC and particle size have been shown to affect the colonic functionality and fermentability. Cell wall materials with a high WHC cause an increase in the volume of gastric contents thereby reducing their passage through the gut and thus the time taken for gastric emptying *(127)*. Thus, fibers with a high WHC are not completely utilised, but are beneficial because they add bulk to the stool in addition to the microbiota and prevent constipation *(5)*.

1.7. Thesis outline

Not much is known on the different polysaccharide populations and interactions between polysaccharides in CRP. In addition, the fermentability of CRP has not been studied. As mentioned, the aim of this thesis is to understand the effect of CRP cell wall polysaccharides and their arrangement in the cell wall network on the *in vitro* fermentability of polysaccharides by human colonic bacteria. The first part of the research involved characterization of cell wall polysaccharides in chicory root pulp **(Chapter 2).** The effect of ensiling on the cell wall polysaccharide network was also described in this chapter. **Chapter 3** reveals the fate of cell wall polysaccharides in CRP during the *in vitro* fermentation of the pulp and the effect of soluble fibers and insoluble fibers on the fermentation of the pulp. The fate of cell wall polysaccharides is also determined during *in vitro* fermentation in the continuous flow model TIM-2 **(Chapter 4)**. Next, the type, levels and efficiency of bacterial enzymes produced during fermentation of CRP in a batch model is determined and the effect of CWP arrangement on enzymatic degradation is explained in **Chapter 5**. Findings of all parts of research are discussed in **Chapter 6**.

1.8. References

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Chapter 2

Structural and water holding characteristics of untreated and ensiled chicory root pulp

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Abstract

Cell wall polysaccharides (CWPs) from chicory root pulp (CRP) and the effect of ensiling on CWP structure to reduce the water-holding capacity (WHC) were studied. Sequential extractions of CRP showed that hot water extraction solubilized arabinan-rich pectin and inulin, each representing 6% of all CRP sugars. A significant amount of pectic sugars (46%) rich in uronic acid from CRP was solubilized by chelating agents. Both dilute alkali extraction, which solubilized branched pectin (14% from CRP), and concentrated alkali extraction, which solubilized hemicellulose dominant in xyloglucans (2.5%) mostly of the XXXG type and mannan (0.9%), from CRP CWPs seemed to influence the WHC of CRP. Alkali-insoluble residue (39% of CRP sugars) mainly comprised cellulose and some branched pectin (17% from CRP). Ensiling reduced the methyl esterification of pectin, caused degradation of homogalacturonan and rhamnogalacturonan, and possibly modified the xyloglucan, mannan, and glucan network, reducing the WHC from 6 mL/g to 3.4 mL/g.

2.1. Introduction

Chicory roots (*Cichorium intybus*) are industrially used for the extraction of inulin, a prebiotic fiber ingredient used in many food applications (*1*). Chicory root pulp (CRP) obtained after inulin extraction is rich in cell wall polysaccharides (CWPs), predominantly pectin (*2, 3*). The use of CRP as a fiber supplement in food applications could be promising since plant fibers have been claimed to contribute to health benefits.

Supplementing foods with fiber from such byproducts requires the understanding of its functional properties, such as water-holding capacity (WHC) and swelling behavior. These physical properties are determined by the molecular structure of the cell wall polysaccharides and the cell wall architecture (*4*).

The constituent monosaccharide composition of chicory root pulp has been studied before (*2, 5*). Differences were found in the degree of esterification, sugar contents, and average molar mass of pectins extracted with acid and those with enzymes, such as cellulases and proteases (*5*). Despite the differences between the pectins obtained by the two treatments, they have in common that they were highly methyl esterified (49–53) and acetylated (12–17). Besides the characterization of extracted pectins from CRP, not much information is present on the interactions between different subpopulations of pectin and other CWPs present in the chicory root cell wall.

Processing of raw pulp materials may cause different waterbinding capacities, as has been illustrated for autoclaving of sugar beet pulp (*6*), grinding of sugar beet pulp and citrus pulp (*7*), and ensiling of potato pulp with inoculants involving degradation of starch and pectin (*8*). This clearly points toward interaction between CWPs, which modulates the water-binding capacity of the material. Different polysaccharides within the cell wall can be studied by sequentially solubilizing polysaccharides with extractants of increasing severity (*9*). The aim of this present research was to characterize the composition of CWPs of CRP and their role toward the WHC of the pulp. Furthermore, the effect of ensiling on the CWP structure and WHC was investigated.

2.2. Materials and Methods

Plant Material. CRP was obtained industrially after extraction of inulin from chicory root with hot water at 80 °C. The pulp was dried at 120 °C and was kindly provided by Sensus B.V. (Roosendaal, The Netherlands). The pulp was milled using a 0.5 mm sieve in a Retsch mill (ZM 200, Retsch, Haan, Germany). Ensiling of wet, unheated CRP (ECRP) was performed at Cosun Food Technology Center (Roosendaal, The Netherlands). A container of 20 L was filled completely with the pulp, and air was evacuated by applying pressure on the pulp before sealing the container. The fermentation by endogenous bacteria was performed at 20–25 °C for 21 days. The ensiled pulp was subsequently heat dried at 120 °C and milled to a particle size of 0.5 mm.

Sequential Extraction of Cell Wall Polysaccharides from Plant Materials. The first treatment involved extracting solids from CRP and ECRP with hot water at 80 °C for 1 h. The substrate:extractant ratio was 1:30 (w/v). The suspension was centrifuged (20 min, 38000g, 20 °C) to obtain the extract. The extraction was repeated until no sugars could be detected in the extract using the phenol sulfuric acid color assay. This check was also performed for other extractions. The extracts were combined and dialyzed against demineralized water using cellulose dialysis membranes (cutoff 12–14 kDa for proteins, Visking, Medicell International, London, UK), freeze-dried, and denoted as hot water soluble solids (HWSS). The final residue obtained was also freeze-dried and denoted as water unextractable solids (WUS).

The WUS was treated with chelating agents (0.05 M EDTA/0.05 M ammonium oxalate) in 0.05 M sodium acetate buffer, pH 5.2 at 70 °C for 1 h. The substrate:extractant ratio was 1:50 (w/v). Next, the suspension was centrifuged (20 min, 38000g, 20 °C) to obtain the extracts. The combined extracts and the residue were dialyzed subsequently against 0.1 M ammonium acetate buffer, pH 5.2, and against demineraliszed water before freeze-drying. The dialyzed extract, denoted as chelating agent soluble solids (CHSS), and the residue, denoted as chelating agent unextractable solids (CHUS), were freeze-dried. The CHUS was further treated with dilute alkali (0.05 M NaOH containing 0.02 M $NaBH_4$) at 0 °C for 1 h. The extracts obtained after centrifugation (20 min, 38000g, 20 °C) were combined and, independently from the residue, neutralized and dialyzed subsequently against 0.05 M sodium acetate buffer, pH 5.2, and demineralized water before freeze-drying. The dialyzed

extract, denoted as dilute alkali soluble solids (DASS), and the residue, denoted as dilute alkali unextractable solids (DAUS), were freeze-dried. The DAUS was finally treated with concentrated alkali (4 M NaOH with 0.02 M NaBH4) at 0 °C for 1 h. The extracts obtained after centrifugation (20 min, 38000g, 20 °C) were combined and, independently from the residue, treated the same as for the dilute alkali extraction. This dialyzed extract was denoted as concentrated alkali soluble solids (CASS), and the residue was denoted as concentrated alkali unextractable solids (CAUS).

Enzyme Treatments of CASS. Hemicellulose digests of CASS were obtained by incubating 2.5 mg of CASS in 1 mL of 10 mM sodium acetate buffer (pH 5.0) with enzymes. Separate enzyme incubations were performed at 40 °C for 24 h using xyloglucan-specific endoglucanase (XEG, EC 3.2.1.151 from *Aspergillus aculeatus*, 2.26U,(*10*)), endoxylanase (X, EC 3.2.1.8 from *Aspergillus awamori* 0.00047U, (*11*)), and endomannanase (M, EC 3.2.1.78 from *Aspergillus niger* 0.046U (*12*)). Following incubation, the enzymes were inactivated at 100 °C for 5 min.

Characterization

Sugar Composition. The total constituent monosaccharide content and composition were determined after prehydrolysis 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 analysed by gas chromatography using inositol as an internal standard (*13*). The automated colorimetric m-hydroxydiphenyl assay was used to determine the total uronic acid (UA) content (*14*). Measurements were performed in duplicate. Overall, the coefficient of variation for the measurement of the sugar composition was below 6%. Rhamnose (Rha) levels were used to calculate the rhamnogalacturonan (RG) backbone content of a fraction assuming the RG backbone consists of Rha:UA of 1:1. The rhamnogalacturonan (RG) content was calculated as RG backbone plus arabinose plus galactose. The homogalacturonan (HG) content was calculated as total UA content minus UA content present in RG backbone (*15*).

Molecular Weight Distribution. High-performance size exclusion chromatography (HPSEC) was performed on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) using three TSK-Gel columns connected in series (4000–3000–2500 SuperAW; 150 × 6 mm). The columns were preceded by a TSK Super AW-L guard column (35 × 4.6 mm). All

columns were from Tosoh Bioscience (Tokyo, Japan). Sodium nitrate (0.2 M) was used as an eluent at a flow rate of 0.6 mL/min. A volume of 20 μL of the sample (2.5 mg/ mL in 0.01 M sodium acetate buffer, pH 5.0) was injected and eluted at 55 °C. Solubles were detected using a refractive index detector, Shodex type RI 101 (Showa Denko, Japan). The software used for acquiring the data was Chromeleon version 7. The molecular mass distribution of polysaccharides was determined using pullulan standards (Polymer Laboratories, Varian Inc., Palo Alto, CA, USA) in the molecular mass range 0.18–790 kDa.

Fructan Content. Samples of 1 mg/mL in 0.05 M sodium acetate buffer, pH 4.7, were treated with 10 μL of inulinase (Fructozyme L, Novozymes, Bagsvaerd, Denmark). The hydrolysis was performed at 50 °C for 18 h. The enzymes were inactivated by boiling for 10 min. After 20 times dilution, 25 μL of the digest was injected into a Dionex ICS 3000 system (Dionex) for high performance anion exchange chromatography (HPAEC) using a Dionex ICS 3000 autosampler. The system was equipped with a Dionex CarboPac PA-1 column (2 × 250 mm) in combination with a Carbopac PA-1 guard column (2 × 50 mm). The system was equipped with pulsed amperometric detection. Fructose, glucose, and saccharose were eluted at 0.3 mL/min using a gradient with 0.1 M NaOH (A) and 1 M NaOAc in 0.1 M NaOH (B): 0–15 min from 100% A to 85% A and 15% B, followed by a washing step for 9 min with 100% B and an equilibration step for 14 min with 100% A. Total fructan concentration (Cf) was calculated using the equation Cf = k(Ff + Gf), in which Ff is total fructose released from the fructans, Gf is total glucose released from the fructans, and k is a correction factor for water uptake of monosugars after hydrolysis. k = [180DP – 18(DP – 1)]/(180DP)(*16*) in which DP is the degree of polymerization.

MALDI-TOF MS. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed for oligomer analysis using an Ultraflextreme workstation (Bruker Daltonics, Bremen, Germany). Mass spectra were obtained in positive mode using a nitrogen laser of 337 nm. After a delayed extraction in a time of 200 ns, the ions were accelerated to a kinetic energy of 12 kV and detected using the reflector mode. The laser intensity was adjusted to obtain clear mass spectra. A minimum of 100 mass spectra were used. Prior to obtaining mass spectra of unknown samples, a series of maltodextrins (mass range of 350–2350 Da) were used for calibration. Samples were desalted using AG 50W-X4 resin, and 1 μL of the desalted sample was added to a dried spot of matrix of 1 μL of 10 mg/ mL 2,5-dihydroxybenzoic acid (Bruker

Daltonics) in 50% (v/v) acetonitrile on the MALDI plate. The sample was dried, and 1 μL of matrix was spread over the dried spot and dried.

Degree of Acetylation and Methyl Esterification. The degree of methyl esterification (DM) and degree of acetylation (DA) of polysaccharides were determined by adding 0.8 mL of 0.4 N sodium hydroxide in 2-propanol/water (50/50 v/v) to 10 mg for 4 h and analysing the acetic acid and methanol released by HPLC (*17*). The DA and DM were calculated as moles of acetic acid or methanol per 100 mol of UA respectively.

Protein Content. The protein content (N × 6.25) was determined on a Thermo Quest NA 2100 nitrogen and protein analyser (Interscience, Breda, The Netherlands) by combustion of the sample. D-Methionine (Acros Organics, NJ, USA) was used for calibration, and cellulose (Fluka, Buchs, Switzerland) was used as a blank.

Water-Holding Capacity. The WHC of the material was determined using Baumann's apparatus (*18*). The apparatus was equipped with a glass filter of porosity level G2 (Duran, Wertheim, Germany). The apparatus was set at 25 °C prior to analysis. A blank reading without the substrate was set as the starting point for measurement. Approximately 10–80 mg of sample was placed on the glass filter, and the amount of water absorbed until saturation was determined. All samples were analysed in triplicate. Evaporation of water over time was measured for a blank filter in triplicate. This loss in water was used to correct the amounts of water held by samples.

2.3. Results and discussion

Carbohydrates in Chicory Root Pulp

The carbohydrate contents and molar sugar compositions of CRP and its extracts obtained by sequential extraction are shown in table 2.1. The carbohydrate content of CRP is 64% w/w. Dominant sugars are uronic acid (UA, 38 mol %), glucose (Glc, 31 mol %), and arabinose (Ara, 15 mol %). The values are in agreement with earlier findings (*2*). The high pectin content makes CRP a good alternative to all cereal-based fibers, especially due to the absence of off flavors compared to other fiber-rich agricultural by-products such as sugar beet pulp. CRP contains pectins that are highly methyl esterified (DM of 70). CRP is highly acetylated (DA of 43), as has been seen before for sugar beet pectin (DA of 35) (*19*). Similar high levels of DM and DA have been reported for chicory root Alcohol Insoluble

Residue (AIR), 68 and 36, respectively (*5*). The protein content (table 2.1) in CRP is 7.6% w/w and is similar to the protein content of chicory root AIR (7.4% w/w) (*5*). About 81% of proteins are recovered in the WUS fraction. The presence of proteins in acid-extracted high-Mw pectins (500 kDa) from chicory root AIR has been reported before (*5*). Yields of carbohydrates extracted sequentially from CRP are shown in table 2.1. HWSS represents only 11% of all carbohydrates present in CRP and indicates a poor extractability of CWP in hot water. CHSS contained 29% of the carbohydrates, while DASS and CASS represented 9% and 4% of the CRP carbohydrate yield, respectively. The final residue represented 39% of the carbohydrates present in CRP.

Table 2-1. Constituent monosaccharide composition of chicory root pulp (CRP) and fractions derived from the pulp

	Carbohydrate yield [g in fraction per100g in pulp]	Mol %								Total Sugars	DM	DA	Protein
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	% w/w	%	%	%w/w
CRP	100	1	0	15	4	4	7	31	38	64	70	43	7.6
HWSS	11	1	0	31	1	10	7	13	37	50	90	27	3.5
WUS	87	1	0	12	4	2	7	33	40	68	70	46	7.5
CHSS	29	1	0	11	0	1	4	1	82	52	49	14	
CHUS	52	1	0	10	6	3	7	53	19	59	52	91	
DASS	9	2	0	31	1	1	17	2	46	52			
DAUS	45	1	0	9	8	3	7	61	11	62			
CASS	4	0	2	5	29	17	7	35	5	56			
CAUS	39	1	0	10	4	1	7	67	10	67			

DM/DA: Degree of methyl/acetyl esterification expressed as moles of methanol esters /acetyl groups per 100 moles uronic acid respectively. Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

HWSS The constituent monosaccharide composition of HWSS revealed (table 2.1) water-soluble pectins rich in both galacturonic acid (37 mol %) and arabinose (31 mol %). Pectin is dominantly present in homogalacturonan (HG) segments, as concluded from the ratio UA:Rha 35:1. Among the pectic sugars present in CRP (GalA, Ara, Gal), 24% of all Ara is soluble in hot water. These water-soluble pectins are highly methyl esterified (DM 90) and

highly acetylated (DA 27). The hot water treatment resulted in the extraction of mannose- and glucose-containing material (table 2.1). Since inulin might be present (*2*), and fructose from inulin is reduced with $NaBH_4$ to produce a mixture of mannitol and glucitol during the analysis of alditol acetates (*20*), the extract was treated with inulinase and analysed by HPSEC (figure 2.1). It clearly showed degradation of a population with an average molecular mass of 3 kDa. This indicated that inulin is present in HWSS and has approximately an average DP of 18. HPAEC analysis confirmed the presence of inulin and DP range (data not shown). The inulin content in this dialyzed extract was determined to be 24.2% w/w, representing 5.2% of all carbohydrates present in CRP carbohydrates. Dialysis did not remove much inulin since inulin in the undialysed extract represented 5.9% of all sugars from CRP. This indicated that industrial extraction removed most of the inulin oligomers with DP ≤ 18 from CRP.

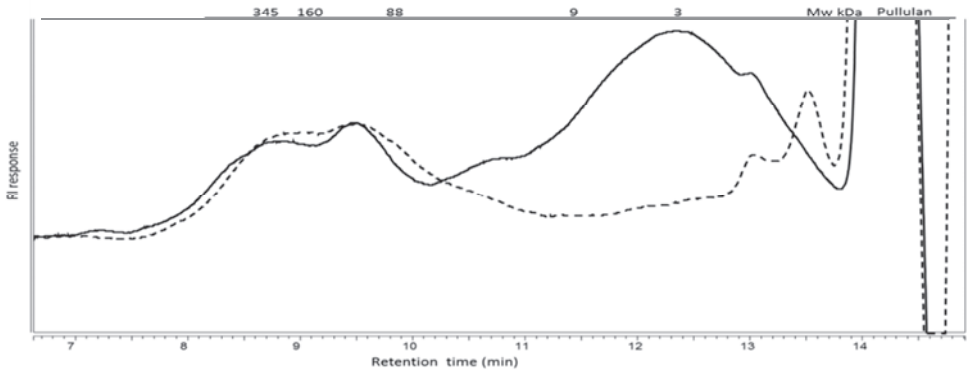

Figure 2-1. HPSEC elution patterns of HWSS before (solid line) and after treatment with inulinase (dotted line)

Sugar Composition and Distribution of CWP in Extracts from WUS

CRP mostly comprises water-insoluble carbohydrates (table 2.1), as 87% of all carbohydrates from CRP were retained in WUS. The WUS contained 68% w/w carbohydrates and was dominant in UA (40 mol %), Glc (33 mol %), and Ara (12 mol %). Pectins in WUS are rich in methyl esters (DM 70) and acetyl groups (DA 46). The extracts obtained from WUS are described below.

CHSS

As the major extract in terms of yield from the insoluble cell wall network, the pectic sugars (GalA+Rha+Ara +Gal) in CHSS constitute 46% of all pectic sugars from CRP. The extract is abundant in HG (82 mol % UA). While representing only 26% of all rhamnogalacturonan (RG) backbones from CRP, 60% of all HG from CRP is represented in CHSS. Ara + Gal side chains constitute only 18% of all Ara +Gal from CRP. Although CHSS pectins are less methyl esterified than HWSS pectins, they still contain a significant amount of methyl esters (DM 49). Besides methyl esterification, CHSS pectins are also acetylated (DA 14).

Following the extraction of calcium-bound pectin by the chelating agent, pectins still containing esters may remain unextracted by the chelating agent in the residue (CHUS). Since sequential alkali extraction will remove methyl esters and acetyl groups, CHUS was analysed for DA and DM (table 2.1). It was found that pectin in CHUS (with only 27% of all UA from CRP) was highly methyl esterified (DM 52). Acetyl groups constituted 57% of all acetyl groups from CRP. If all the acetyl groups were assumed to be present on pectin, the DA could be estimated as 91. This is quite high, but possible (*21*). Nevertheless, it should be taken into account that some nonpectic CWPs also bear acetyl groups (*22*).

DASS

DASS is a minor fraction, rich in branched pectin compared to the other extracts. It represents 14% of the CRP pectic sugars. An increased proportion of RG backbone over HG (HG:RG backbone 9) compared to CHSS (HG:RG backbone 30) and higher branching of the RG backbone with side chains of Ara and Gal (Ara+Gal/Rha 21) compared to CHSS (Ara+Gal/Rha 11) are seen. Among the side chains, the Gal:Ara ratio is higher in DASS (0.6) than in CHSS (0.4) and HWSS (0.2). This increase in Gal:Ara ratio with extraction severity indicates an increase in pectin complexity containing galactose in the entangled network of pectins.

CASS

Upon extraction with 4 M alkali, CASS was found to mainly contain hemicellulose, as seen from glucose (35 mol %), xylose (29 mol %), and mannose (17 mol %). Hemicelluloses from CRP have not been studied before. The xylose:glucose ratio in CASS is 0.8. The ratio of xylose:glucose of 0.75 is indicative of xyloglucan of the XXXG type in which three out of four glucose units (G) are substituted with xylose units (X) (*23*).

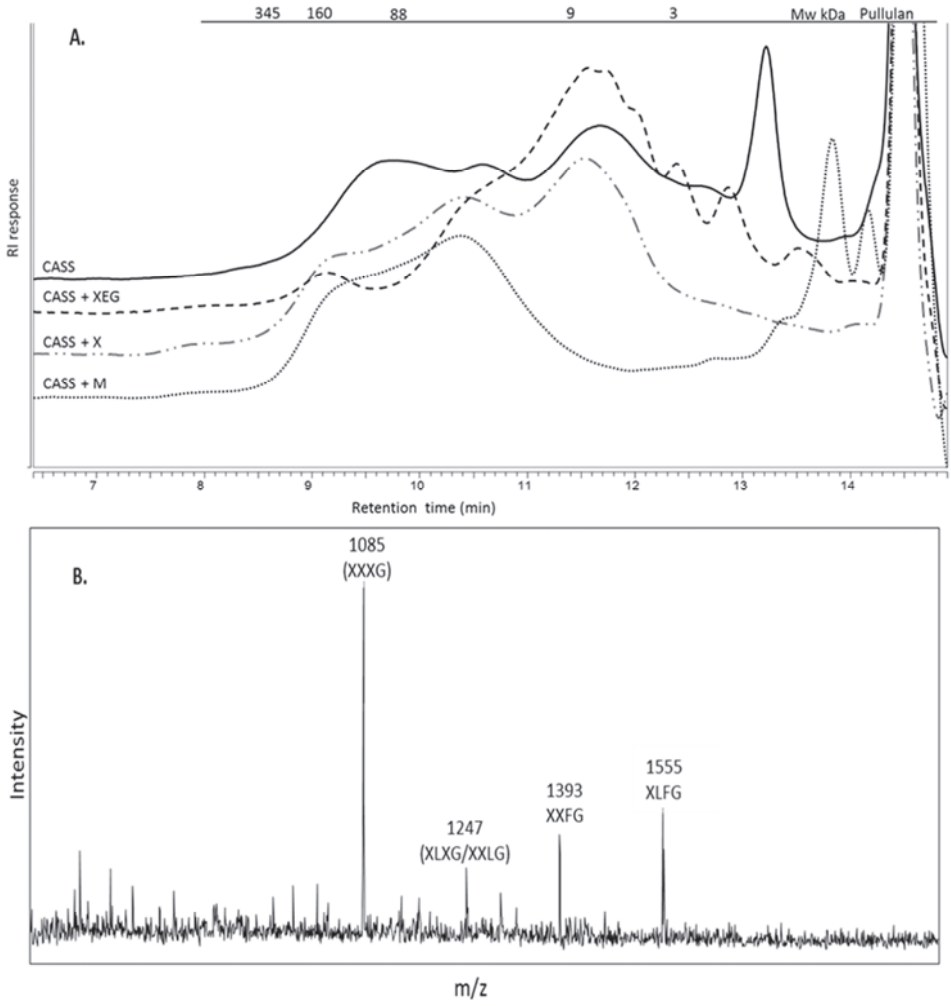

Figure 2-2. **A.** HPSEC elution pattern of CASS from chicory root pulp (CRP) treated with xyloglucan specific endoglucanase (XEG), xylanase (X) and mannanase (M), **B.** MALDI-TOF mass spectrum of xyloglucan oligomers obtained after XEG treatment of CASS

Although indicative for xyloglucans, the ratio of xylose:glucose of 0.8 cannot rule out the presence of xylan. The presence of XXXG-type xyloglucan in CRP CASS was confirmed by digestion with xyloglucan-specific endoglucanase. HPSEC analysis showed degradation of the high molecular mass material after digestion with XEG (figure 2.2A). Oligosaccharides formed upon degradation of xyloglucan were identified by the mass to

charge ratios of their sodium adducts with MALDI-TOF MS (figure 2.2B). The spectrum showed four peaks corresponding to sodium adducts of XXXG (m/z = 1085) as the major peak, XLXG or XXLG (m/z = 1247) in which L corresponds to XXXG substituted with Gal, and XXFG (m/z = 1393) and XLFG (m/z = 1555) in which F corresponds to XLXG or XXLG further substituted with fucose (Fuc). Gal and Fuc decorations on xyloglucan were also indicated from the sugar composition of the extract: Gal (7 mol %) and Fuc (2 mol %). Apart from xyloglucans, the presence of mannans in CASS was indicated from the sugar composition. Digestion of CASS with mannanase or xylanase showed degradation of the material with apparent molecular masses of 9 and 3–160 kDa, respectively (figure 2.2A). These digestions further indicate the presence of mannans and xylans in CASS. This was confirmed from HPAEC and MALDI-TOF MS, which indicated the presence of hexose oligomers for mannans and pentose oligomers substituted with 4-O-methylglucuronic acid for xylans (data not shown).

CAUS

The residue retained as the major fraction after 4 M alkali treatment is rich in Glc (67 mol %); 87% of all Glc from CRP is retained in the residue. Although only 4 mol % xylose (Xyl) is present, 44% of all Xyl from CRP is present in CAUS. This residual Xyl could arise from xylans or xyloglucans bound to cellulose microfibrils that remain unextracted with concentrated alkali (*10, 24, 25*). Next to cellulose, this fraction still contains some RG branched with Ara and Gal side chains (Ara+Gal/Rha 18), as also seen for DASS pectins. CAUS contains 30% of all Ara+Gal present in CRP, thereby constituting a significant proportion of Ara- and Gal-rich pectin. Most of the Gal is believed to originate from pectin side chains, since xyloglucans bearing Gal decorations would be present in minor levels due to the low content of Xyl (4 mol %). Side chains of Ara and Gal may play a role in anchoring pectins to cellulose microfibrils through covalent bonds (*26*).

WHC of Residues Obtained from Sequential Extractions

In order to understand how CWPs and their network contribute to the WHC of CRP, residues obtained after sequential extractions of CWPs were analysed for their WHCs (Figure 2.3). The WHC for CRP was 6 mL/g. This value is in the same range as found for other pulp materials, such as sugar beet pulp (5.5 mL/g) (*27*). The WHCs for WUS, CHUS, DAUS, and CAUS were 13, 14, 22, and 13 mL/g, respectively. Despite recovering most of CRP polysaccharides in WUS (87% of CRP PS), the increase in the WHC of WUS can be

due to an increase in the porosity of the cell wall caused by hot water treatment (*28, 29*). Removal of 35% of WUS CWPs by the chelating agent did not alter the WHC of the network, as seen from similar values of WHC for WUS and CHUS. Thus the removal of HG-rich pectin has no influence on the WHC of CRP. The high WHC for DAUS, despite representing still 87% of CWPs from CHUS, could arise from a different arrangement of CWPs in the network due to removal of branched pectins (DASS). Although such a network is abundant in cellulose, it contains hemicellulose and still some branched pectin, which may be important for hydration. Since the residue has been subjected to dilute alkali, esters lost from polysaccharides may have altered the WHC of the network. Removal of even minor amounts of CASS CWPs (representing only 9% of DAUS CWPs) resulted in a much lower WHC for CAUS compared to DAUS. CASS CWPs dominant in xyloglucan followed by mannan-rich hemicellulose (representing 2.5% and 0.9% of all CRP CWPs, respectively) possibly suggest their positive role in the WHC of DAUS. In addition, entrapped xyloglucans have been found to show motional properties intermediate between cellulose and pectin (*30*). Thus, they may contribute to WHC when bound to cellulose microfibrils.

The observed WHC for CAUS may be an overestimation because of alkali-swollen cellulose (*31, 32*).

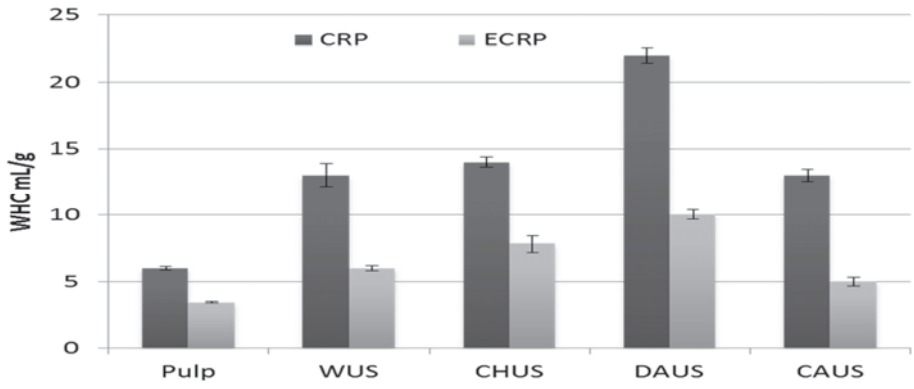

Figure 2-3. Water holding capacity (WHC) of chicory root pulp (CRP) and ensiled CRP (ECRP) and fractions derived from the pulps where WUS:water unextractable solids, CHUS: chelating agent unextractable solids, DAUS: dilute alkali unextractable solids, CAUS: concentrated alkali unextractable solids.

Effect of Ensiling on the Cell Wall Network of CRP

Ensiling of CRP was performed to determine if such a treatment could reduce the WHC of the pulp. A significant reduction in the WHC of 44% from 6 mL/g to 3.4 mL/g was indeed seen (figure 2.3). In order to determine the role of CWPs and their network toward the WHC of the pulp, ensiled chicory root pulp was further characterized. Analysis of the sugar composition of ECRP (table 2.2) showed that it was quite similar to that of CRP (table 2.1). However, ensiling caused demethyl esterification, as observed by the lower DM (22) of ECRP compared to CRP (70). The acetylation of CWPs remained similar to that of CRP. Despite the similarity in sugar composition and acetylation, possible modifications on the cell wall network caused by ensiling might have caused a reduction in the WHC. Hence, sequential extractions were performed on ECRP.

Table 2-2. Constituent monosaccharide composition of ensiled chicory root pulp (ECRP) and fractions derived from the pulp

	Carbohydrate yield	Mol %								Total Sugars	DM	DA	Protein
	[g in fraction per100g in pulp]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	% w/w	%	%	%
ECRP	100	2	0	15	4	3	7	31	38	56	22	49	9.2
HWSS	39	2	0	35	0	3	10	3	47	72	24	36	3.1
WUS	55	1	0	5	7	3	5	56	21	55	6	79	10.4
CHSS	9	1	0	5	0	1	4	1	88	43	6	8	
DASS	2	3	0	19	4	3	26	14	31	33			
CASS	8	0	2	7	28	15	10	31	7	54			
CAUS	35	1	0	3	4	1	4	83	5	59			

DM/DA: Degree of methyl/acetyl esterification expressed as moles of methanol esters /acetyl groups per 100 moles uronic acid respectively. Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

Effect of Ensiling on Solubility of the CWP Network and WHC

Ensiling of CRP increased the hot water extractability of CWPs. This was indicated (table 2.2) by the higher carbohydrate yield (39%) for ECRP HWSS compared to CRP HWSS (11%) (table 2.1). Dominant among the sugars solubilized in ECRP HWSS are pectic sugars, as seen from the sugar composition (table 2.2). ECRP HWSS had 4 times more pectic sugars than in CRP HWSS (figure 2.4). This proves that ensiling increased the extractability of pectic sugars from the insoluble network of CRP, which are extractable

only under more severe conditions for CRP (figure 2.4A). The pectins in ECRP HWSS were shown to be degraded during ensilage, as can be seen from the elution profile by HPSEC (figure 2.4A). The dominant population of low molecular mass (Mw) less than 3 kDa indicated that dialysis was not sufficient to remove soluble material of low molecularmass. Removal of higher proportions of pectin from the cell wall network and reduced Mw and DM of pectin could explain a lower WHC for ECRP WUS (6 mL/g) compared to CRP WUS (13 mL/g). Furthermore, the characteristics of the residual CWPs in the insoluble network may also contribute to a lower WHC for ECRP WUS.

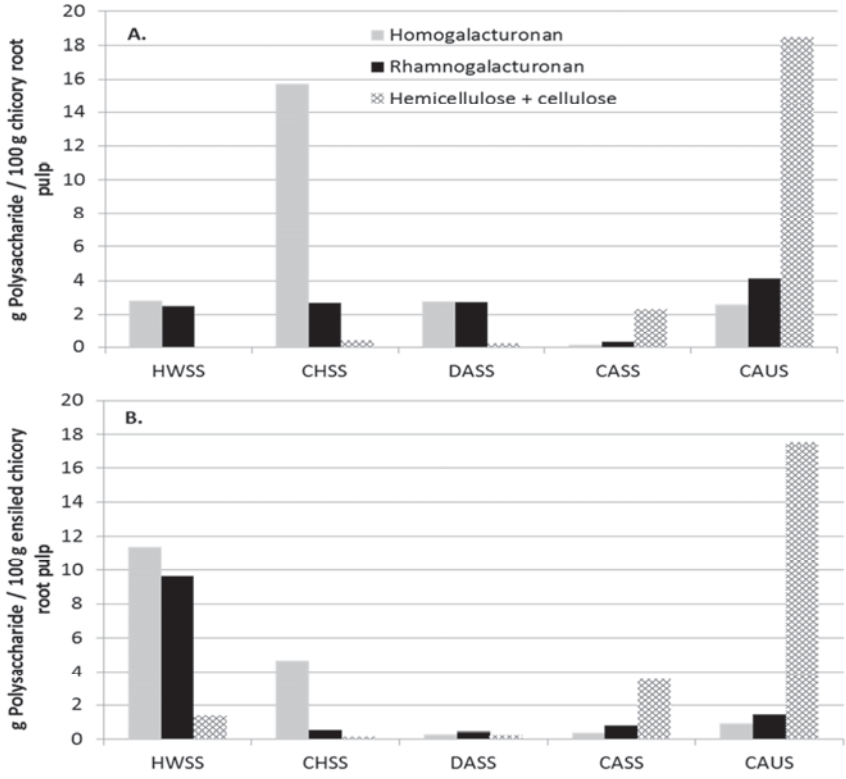

Figure 2-4. Distribution of polysaccharides in fractions derived from **A.** 100 g of chicory root pulp (CRP) and **B.** 100 g of ensiled chicory root pulp (ECRP)

Effect of Ensiling on the Extractability of CWPs and WHC

Fractions obtained from the insoluble cell wall network (WUS) of ECRP had lower WHCs compared to those from CRP (figure 2.3). Ensiling increased the extractability of HG by 4 times in HWSS than CRP HWSS, having the consequence of a 4 times lower HG recovery

in ECRP CHSS compared to CRP CHSS (figure 2.4). HPSEC analysis of the extracted HG-rich pectin (88 mol % UA) in ECRP CHSS pointed to a lower Mw compared to CRP CHSS (figure 2.5B). Thus, increased removal of HG from WUS and degradation of residual HG-rich pectin in WUS during ensiling lowered the WHC of ECRP WUS (6 mL/g) compared to CRP WUS (13 mL/g). ECRP CHUS (sum of DASS, CASS, and CAUS) contains 3 times less RG and 4 times less HG than the fractions in CRP CHUS (figure 2.4). Ara-rich pectin was degraded in CHUS during ensiling, as indicated from low Ara:Rha in ECRP DASS (7:1) and CAUS (5:1) (table 2.1) compared to CRP DASS (13:1) and CAUS (11:1).

The lower proportion of HG and RG and degraded Ara-rich pectin reduced the WHC of ECRP CHUS (7.8 mL/g) compared to CRP CHUS (14 mL/g) (figure 2.3). Furthermore, the WHCs for ECRP DAUS (10 mL/g) and CAUS (5 mL/g) were lower compared to CRP DAUS (22 mL/g) and CAUS (13 mL/g) (figure 2.3), respectively. ECRP CAUS derived from ECRP DAUS contains 3 times less pectic sugars than CRP CAUS (figure 2.4). Other polysaccharides, such as xyloglucan, glucan, and mannan were also modified in ECRP CAUS, increasing their extractability in ECRP CASS, as indicated from a higher carbohydrate yield (table 2.2) compared to CRP CASS and similar sugar composition to CRP CASS (table 2.1).

2.4. Conclusions

Studies on WHC showed that the WHC of chicory root pulp may change depending on the arrangement of cell wall polysaccharides within the plant cell wall network. The removal of branched pectin seemed to influence the WHC strongly. Besides, alkali-soluble xyloglucan and mannan-rich hemicellulose were indicated to contribute positively to the WHC. Ensiling degraded arabinose-rich pectin, homogalacturonan, and rhamnogalacturonan and modified the network consisting of xyloglucan, mannan, and glucan, all of which caused a significant reduction in the WHC. Following the characterization of chicory root pulp before and after ensiling, the use of CRP as a fiber supplement may have become closer to application. We now know that small changes in architecture as a result of mild processing or even by a targeted enzyme treatment will enable modulation of the WHC of CRP, resulting in improved properties of the CRP in the final fiber-rich product.

2.5. Acknowledgements

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Chapter 3

The effect of soluble and insoluble fibers on the fermentation of chicory root pulp

Submitted for publication

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Abstract

The aim of this research was to study the *in vitro* fermentation of chicory root pulp (CRP) and ensiled CRP (ECRP) using human faecal inoculum. Analysis of carbohydrate levels in fermentation digests showed that 51% of all CRP carbohydrates were utilized after 24 h of fermentation. For ECRP, having the same cell wall polysaccharide composition as CRP, but with solubilisation of four times more of CRP pectin due to ensiling, the fermentation was quicker than CRP as 11% more carbohydrates were utilized within the first12 hours. The level of fiber utilization for ECRP after 24 h was increased by 8% compared to CRP. This effect on fiber utilization from ECRP seemed to arise from i) increased levels of soluble pectin fibers (arabinan, homogalacturonan and galactan) and ii) a more hypothesized open structure of the remaining cell walls in ECRP which was more accessible to degradation than the CRP cell wall network.

3.1. Introduction

Dietary fiber (DF) includes cell wall polysaccharides (CWPs), such as pectin, hemicellulose and cellulose, which are resistant to absorption or digestion in the human small intestine and become available for (partial) fermentation in the colon by the human microbiota (*1*). Some types of DF have been reported to contribute to gut health. Pectin has been shown to reduce adhesion of pathogens to intestinal epithelial cells (*2*), reduce ammonia generation (*3*), induce intestinal mucosal proliferation and maintain intestinal integrity (*4*). Fermentation of DFs in the colon promotes the growth of microbiota. Short chain fatty acids (SCFA's) produced by fermentation of DF entering the proximal colon, lower the pH of the proximal colon and inhibit the growth of pathogens (*5*). While the availability of DF in the proximal colon is beneficial, the absence of DF in the distal colon may lead to a switch to protein fermentation by the microbiota, which amongst others leads to the production of ammonia, and thereby leads to a pH that is near neutral (*6, 7*). Such conditions make the distal colon vulnerable to chronic gut disorders (*8, 9*). Thus, it would be beneficial to use a mixture of DFs, part of which are fermented in the proximal colon and part of which remain available for further fermentation in the distal colon. Research on the *in vitro* fermentability of pectin-rich agricultural by-products, such as sugar beet pulp, has been performed using human faecal inoculum. More than 80% of the initial carbohydrates in sugar beet pulp were utilized within 24 h of fermentation indicating high fermentability of the pulp (*10*). Furthermore, the fermentability may be influenced by processing resulting in a modified cell wall network as has been shown for autoclaved sugar beet pulp (*10*). Chicory root pulp (CRP) obtained after inulin extraction is rich in CWPs (58% w/w), predominantly pectin (62%), followed by cellulose (27%) and hemicellulose (11%) as DFs (*11*). In addition to CWPs, CRP contains (6% w/w) residual levels of the storage polysaccharide, inulin as a DF (*11*). The use of CRP as a DF supplement in food applications could thus be promising. While abundant in insoluble fibres of different populations of CWPs, the fermentability of CRP has not been studied. Processed CRP, such as ensiled CRP (ECRP), has a loosened cell wall network with a reduced water holding capacity (WHC) (3.4 mL/g) compared to CRP (6 mL/g) and contains four times more soluble pectin fibers than CRP (*11*). Such a modification of the cell wall

network with more soluble fibers may have an effect on the extent and rate of fermentation of CRP. Thus, the aim of this research was to determine the effect of processed and unprocessed cell walls of CRP on the *in vitro* fermentability of the pulp using human faecal inoculum.

3.2. Materials and Methods

CRP and ECRP were obtained from Cosun Food Technology Center (Roosendaal, The Netherlands) and have been described previously (*11*). Since they contain dietary fibers and no starch and no digestible oligosaccharides that would be digested in the stomach or the small intestine, no pre-digestion was performed on these materials.

In vitro fermentation:

The fermentation medium used in this study was modified standard ileal efflux medium (SIEM) according to Ladirat et al. (*12*) Tween 80 was omitted from the fermentation medium because it influenced analysis of polysaccharide degradation products. All medium components were purchased from Tritium Microbiology (Veldhoven, The Netherlands). The pH of the fermentation medium was adjusted to 5.8, simulating the pH in the proximal colon. The inoculum was a standardised pool of adult faecal inoculum according to the validated protocol described elsewhere (*13*) and prepared from eight healthy European adults (25-45 years old) who did not receive antibiotics during 2 months before donation. The inoculum (6 mL) was incubated in the modified SIEM (54 mL) for activation (*12*).

CRP and ECRP (100 mg) were added to 9 mL of the modified SIEM in 25 mL bottles for each time point. The activated inoculum (1 mL) was added to the fermentation medium and flushed with CO2. The bottles were immediately closed with rubber stoppers and sealed with aluminium caps. Fermentations were performed in duplicate for each substrate at 37 °C and shaken at 300 rpm until 24 h. A blank with only microbiota was taken as a control.

Fermentation digests were inactivated at 100°C for 5 min and split into two parts. One part was reserved for direct analysis and the other part of the fermentation digest was freeze dried for further characterization. Part of the dried fermentation digest was suspended in millipore water (10mg/mL) and centrifuged (10 min, 9,000 x g, 4°C) to obtain soluble and insoluble fractions. The insoluble fraction was freeze dried and used along with the original

dried fermentation digest for analysing constituent monosaccharide content and composition. Constituent monosaccharide content in the soluble fraction was calculated from the difference in individual monosaccharide contents between the dried fermentation digest and the dried insoluble fraction of the fermentation digest.

Analytical methods

Constituent monosaccharide composition.

The constituent monosaccharide content and composition were determined using a pre-hydrolysis step with 72 (w/w%) sulphuric acid at 30°C for 1 h followed by hydrolysis with 1 M sulphuric acid at 100°C for 3 h. The monosaccharides formed upon hydrolysis were derivatized to alditol acetates and analysed by gas chromatography using inositol as an internal standard (*14*). The colorimetric m-hydroxydiphenyl assay (*15*) was used to determine the total uronic acid (UA) content.

Molecular weight distribution

Fermentation digests were centrifuged (10min, 18,000 x g, 24°C) to obtain the soluble fraction which was analysed for molecular weight distribution using High Performance Size Exclusion Chromatography (HPSEC) on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) as described before (*11*). Three TSK-Gel columns in series (4000-3000-2500 SuperAW; 150 x 6 mm) were used for the analysis. They were preceded by a TSK Super AW-L guard column (35 x 4.6mm). All columns were from Tosoh Bioscience (Tokyo, Japan). The molecular weight was estimated based on a standard curve of pullulan standards.

Oligosaccharide profiling

High Performance Anion Exchange Chromatography (HPAEC) was performed on a 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 50 mm). Ten μL of the soluble fraction of the fermentation digests (diluted twice with millipore water) was injected into the system using a Dionex ICS5000 autosampler. The system was equipped with pulsed amperometric detection. The flow rate was 0.3mL/min and the gradient used was as

described elsewhere (*16*). The software used was Chromeleon version 7 (Dionex). Oligomers of galacturonic acid (DP 1 to DP 5) were used as standards to identify and quantify galacturonic acid oligomers in the elution profile.

Analysis of short chain fatty acid (SCFAs) and organic acids

SCFAs and organic acids (lactate and succinate) were analysed as described elsewhere (*17*).

Microbiota composition

Samples from a twin batch fermentation of CRP were analysed for microbiota composition. The chemical analyses of fermentation of CRP (SCFA production, monosaccharide utilization) are correlated to the microbiota composition. The microbiota composition for ECRP fermentation was not performed due to limitations in the sample amount. The microbiota compositions for 2 independent duplicates of a 9 h fermentation digest of CRP (supplementary figure 3.S1) were similar indicating reproducibility for fermentations of the cell wall material.

Molecular techniques

DNA from the fermentation digests was isolated using the AGOWA mag Mini kit (DNA Isolation Kit, AGOWA, Berlin, Germany), according to the manufacturer's instructions.

Generation of the PCR amplicon library was performed by amplification of V5-V7 hypervariable region of the small subunit ribosomal DNA gene (16S rDNA). All samples were analysed as described elsewhere (*18*).

3.3. Results and discussion

Characteristics of fibers in chicory root pulp and ensiled chicory root pulp

Table 1 shows the constituent monosaccharide composition of chicory root pulp (CRP) and ensiled CRP (ECRP) (*11*). CRP is abundant in carbohydrates (64 w/w%) of which 58 w/w% represents cell wall polysaccharides (CWPs) and 6 w/w% represents minor levels of the storage polysaccharide inulin with an average DP of 18 (*11*). The CWPs calculated from the mol% monosaccharide composition and total sugar content (w/w %) (table 3.1) contain 62 w/w% pectin (uronic acid + rhamnose + arabinose + galactose), 27% cellulose

(arising from glucose not present in xyloglucan) and 11% hemicellulose (xylose + mannose + fucose + glucose in xyloglucan) as characterised earlier (*11*).

Table 3-1. Constituent monosaccharide composition of of chicory root pulp (CRP) and ensiled chicory root pulp (ECRP)

	Mol %								(w/w %)	Carbohydrate yield [g in fraction per100g in CRP/ECRP]
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total	
CRP	1	0	15	4	4	7	31	38	64*	100
CRP Soluble	1	0	27	1	13	6	19	33	50	12
CRP Insoluble	1	0	12	4	2	7	33	40	68	87
ECRP	2	0	15	4	3	7	31	38	56	100
ECRP Soluble	2	0	27	0	3	8	3	57	62	48
ECRP Insoluble	1	0	5	7	3	5	56	21	55	55

Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA), * contains 6 w/w % inulin. Results from an earlier study (*11*).

The carbohydrates present in the soluble fraction represent only 12% of the CRP carbohydrates. As determined earlier (*11*), half of the soluble fraction is arabinose rich pectin and the other half is inulin. 87% of the carbohydrates from CRP are insoluble and have similar polysaccharide composition as the total CRP (table 3.1). The ECRP is rather similar in composition to CRP (table 3.1), but contains no inulin (*11*).

As indicated by the higher carbohydrate recovery (45%) in the soluble fraction from ECRP compared to that from CRP (12%) (table 3.1), ECRP has more soluble fibers than CRP. Dominant among the sugars solubilised are the pectic sugars as seen from the sugar composition (table 3.1). As determined from an earlier study (*11*), the soluble fraction of ECRP represents 4 times more pectic sugars than that from CRP. The pectic sugars from the insoluble network of CRP are only extractable under severe conditions, like chelating agents and alkali (*11*). Apparently, they can be solubilised by ensiling. As a consequence, the insoluble fraction of ECRP represents a lower recovery of carbohydrates (55%) compared to CRP (87%) and is enriched in (hemi)cellulose (table 3.1).

Fermentation of CRP and ECRP - short chain fatty acid production

CRP and ECRP were well fermented as indicated by the increase in levels of short chain fatty acid (SCFAs) produced (mmol /g substrate) in time (figure 3.1) as well as from the decrease in CWP content discussed later in this study.

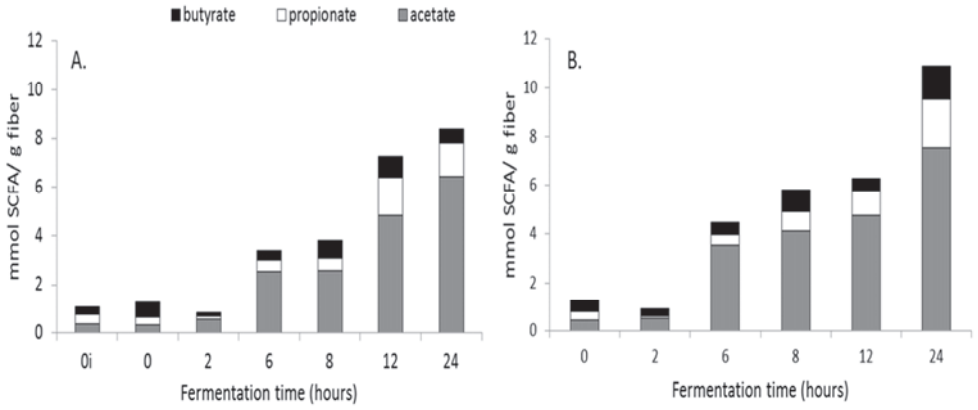

Figure 3-1. Short chain fatty acids production during fermentation of **A:** Chicory root pulp (CRP) (left) and **B:** Ensiled chicory root pulp (ECRP) (right). Succinate and lactate levels are not shown due to low levels (< 0.1 mmol/g fiber). 0i, inoculum + medium. Values present means of duplicate measurements. Standard deviations of the values are presented in table 3.S1 (supplementary data).

The SCFA levels at the start of fermentation (0 h) for CRP and ECRP arose from the inoculum added to the medium as seen from similar levels between the inoculum blank (0i) and 0 h for CRP and ECRP fermentation. The initial SCFA levels (0 h) for both CRP and ECRP are higher than found for the digests after 2 h of fermentation indicating utilization of SCFAs during the first 2 h. After 24 h of fermentation of CRP, 8.4 mmol SCFAs /g substrate was present. Other pectin rich fiber materials, such as sugar beet pulp and citrus pulp, when fermented for 24 h using human faecal inoculum contained 3.4 and 4.8 mmol SCFAs /g substrate organic matter, respectively (*19*).

Compared to CRP fermentation (figure 3.1A), ECRP fermentation was quicker as high levels of SCFAs were produced within a short time period (6 h) of fermentation (figure 3.1B). Besides a faster ECRP fermentation, a higher SCFA production (10.9 mmol/g) after 24 h of ECRP fermentation was seen compared to CRP fermentation (8.4 mmol/g). This indicated that the modified cell wall network of ECRP with a higher ratio of soluble: insoluble polysaccharides (0.8) (table 3.3) compared to CRP (ratio 0.3) (table 3.3) is more

fermentable than that of CRP. The ratio of acetate: propionate: butyrate was similar between CRP (77:16:6) and ECRP (69:18:13) after 24 h of fermentation. Taking into account the similar carbohydrate composition of CRP and ECRP, this suggested that fermentation of an open cell wall network has limited influence on the utilization by the microbiota. This similarity in the SCFA ratio has also been observed for fermentations of sugar beet pulp and autoclaved beet pulp (*10*).

Minor levels of intermediate fatty acids (*20*), lactate and succinate (<0.1 mmol/g), were observed throughout the fermentation period. The fermentation of higher oligomers of inulin (discussed later) indeed resulted in very little butyrate and lactate formation. Fermentation of higher oligomers of inulin (DP >20) by the microbiota has been reported to hinder the production of butyrate and lactate than short oligomers of inulin (DP < 10) (*21*, *22*).

Molecular weight distribution of soluble fibers

HPSEC elution profiles of solubles from CRP and ECRP fermentation digests are shown in figure 3.2. For CRP fermentation, an increase in the amount of soluble material corresponding to high molecular weight (Mw) (8 – 10 min; 47- 788 kDa) was seen after 2 h of inoculation. From 6 h onwards, an almost complete disappearance in the high Mw fraction suggested degradation/ utilization of the high Mw polysaccharide. This implies either rapid utilization of fibers solubilised during fermentation or direct utilization of insoluble fibers directly by the microbiota system.

Compared to the elution profile of CRP fermentation blank (0 h), the elution profile of the ECRP blank showed a 52% higher amount of high Mw material, as expected from the higher pectin solubility(*11*). This material is present in two populations, (8–9 min; 212-788 kDa) and (9-10 min; 47-212 kDa). After 6 h of fermentation, a decrease is seen in the intensity of both peaks indicating degradation of both the populations. These populations are almost completely utilized after 8 h of fermentation. As for CRP fermentation, almost no high Mw solubles could be seen after 12 and 24 h of ECRP fermentation.

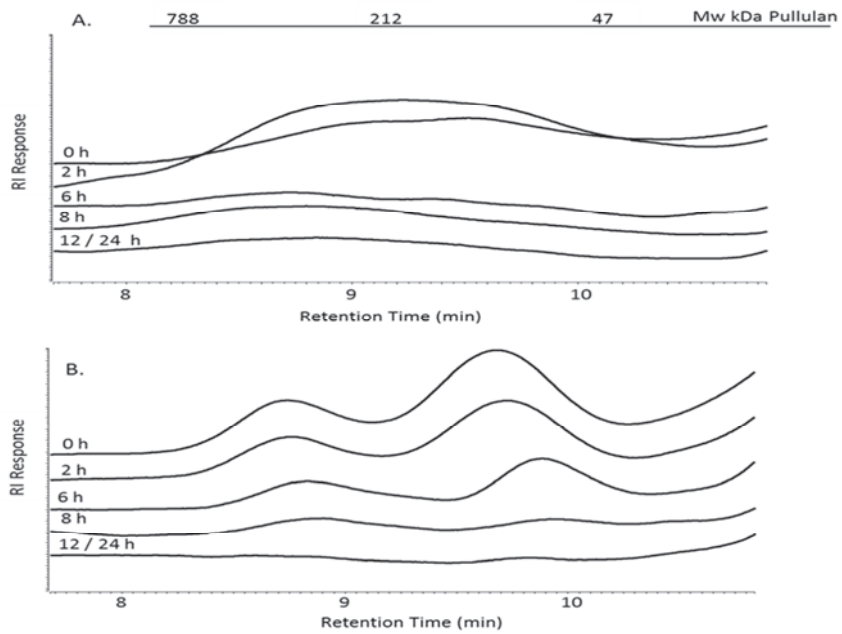

Figure 3-2. HPSEC elution patterns of fermentation digests during fermentation of **A.** Chicory root pulp (CRP) and **B.** Ensiled chicory root pulp (ECRP). Pullulan was used as a calibrant.

Oligomer utilization

In order to determine oligomers formed/utilized during CRP and ECRP fermentation, soluble fractions from fermentation digests were analysed using HPAEC (figure 3.3). Before the start of CRP fermentation (0 h), higher oligomers of inulin (average DP of 18) (*11*) were observed. Since short oligomers of inulin were removed by the industrial extraction from the root, the pulp only contains higher oligomers of inulin, with an average DP of 18. Within 6 h, inulin is almost completely utilized (*11*).

During fermentation, CWPs are degraded to lower Mw material and in a previous study using other sources for CWPs than CRP, intermediate oligosaccharides could be clearly recognised (*16*). However, in this study, no oligomers formed from CWPs were observed during CRP fermentation. *Bacteroides sp.*, becoming dominant during CRP fermentation, as discussed later, are known to be motile and to possess 'specialized polysaccharide utilization systems' in which cell surface proteins are involved in binding to different polysaccharides and transporting the oligomers that are formed directly over the bacterial

membrane (*23*, *24*). Such systems likely prevent diffusion of oligomers. Another possibility for the absence of oligomers could arise from rapid cross feeding of oligomers by other fermenting bacteria (*25*).

Before the start of ECRP fermentation (0 h), a number of peaks can be identified as oligomers of galacturonic acid (GalA) based on standards of GalA oligomers used (figure 3.3B). These oligomers represent approximately 14% of total ECRP GalA and arose from ECRP after the process of ensiling. These oligomers are shown to decrease within the first 6 h of ECRP fermentation pointing to their utilization. Similar to the CRP fermentation, no new oligomers are formed.

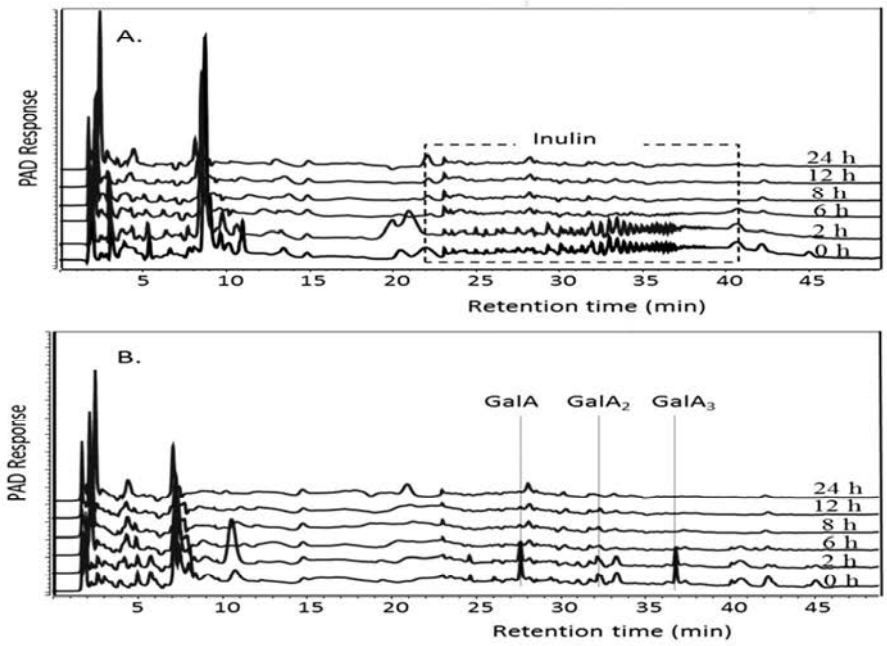

Figure 3-3. HPAEC elution profile of fermentation digests during fermentation of **A.** Chicory root pulp (CRP) and **B.** Ensiled chicory root pulp (ECRP). Legend: Galacturonic acid (GalA), $(GalA)_2$: GalA dimer, $(GalA)_3$: GalA trimer.

Changes in CRP fiber composition during fermentation

Direct utilization of CWPs during CRP fermentation can be revealed from the decrease in the total carbohydrate content as well as from the constituent monosaccharides during fermentation.

The constituent monosaccharide compositions of CRP fermentation digests are shown in table 3.2. Low concentrations (w/w %) of carbohydrates in all the fermentation digests are due to the presence of high concentrations of other fermentation media components, such as proteins and salts *(12, 13)*. The constituent monosaccharide composition of the CRP fermentation blank (0 h) (table 3.2) is similar to that of CRP (table 3.1) indicating that the carbohydrates present in the medium before fermentation do not contain carbohydrates other than from CRP fibers. After 24 h of fermentation, 51% of CRP total carbohydrates were utilized (figure 3.4A).

Table 3-2. Residual carbohydrate levels and constituent monosaccharide composition of digests obtained from *in vitro* fermentation of chicory root pulp (CRP) and ensiled CRP (ECRP)

	Fermentation time (hours)	Residual carbohydrate levels (mg from 90 mg starting material)	Mol %								(w/w %)
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total
CRP	0 (blank)	56	2	0	14	4	5	8	35	32	7
			0.00	*0.05*	*0.69*	*0.25*	*0.67*	*0.10*	*0.36*	*0.19*	*0.00*
	2	46	2	0	14	4	5	8	35	32	7
			0.03	*0.00*	*0.05*	*0.06*	*0.02*	*0.05*	*0.06*	*0.12*	*0.04*
	12	42	2	0	14	5	3	8	35	34	7
			0.01	*0.00*	*0.00*	*0.02*	*0.02*	*0.01*	*0.11*	*0.29*	*0.44*
	24	28	2	0	4	8	4	6	65	10	4
			0.15	*0.03*	*0.02*	*0.55*	*0.08*	*0.13*	*0.50*	*0.25*	*0.04*
ECRP	0 (blank)	55	2	0	14	4	3	8	35	34	7
			0.00	*0.00*	*0.06*	*0.01*	*0.03*	*0.01*	*0.32*	*0.03*	*0.32*
	2	48	2	0	15	4	3	8	36	32	7
			0.20	*0.02*	*0.10*	*0.16*	*0.38*	*0.35*	*1.55*	*2.43*	*0.29*
	12	35	2	0	6	6	4	7	53	22	5
			0.25	*0.06*	*0.69*	*0.50*	*0.36*	*1.17*	*0.02*	*1.43*	*0.71*
	24	22	2	0	2	9	5	5	68	10	4
			0.43	*0.04*	*0.10*	*0.33*	*0.04*	*0.41*	*0.27*	*1.08*	*0.22*

Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA). The values presented are means of duplicate measurements, with standard deviation in italics.

This is rather similar to the degradability of sugar beet fiber showing 60% carbohydrate utilization after 24 h of fermentation with human faecal inoculum *(26).*

Until 12 h of fermentation, the constituent monosaccharide composition remained similar although 25% of all carbohydrates were utilized. This indicated unspecific utilization of CWPs in the first 12 h of fermentation. This is also seen in figure 3.4A from 0 to 12 h of fermentation. As the fermentation continued from 12 h to 24 h, the carbohydrate content was further reduced by another 25% and the constituent monosaccharide composition changed considerably. This suggests a more specific utilization of CWPs from 12 h onwards. The calculated decrease in arabinose (Ara), galactose (Gal), and GalA by 86%, 63%, and 84% respectively, after 24 h (figure 3.4A) indicated enhanced utilization of arabinan, galactan and homogalacturonan (HG) from CRP respectively. The unexpected decrease in glucose utilization from 12 to 24 h (figure 3.4A) might arise from an increase in bacterial cell walls containing glucose (*27*).

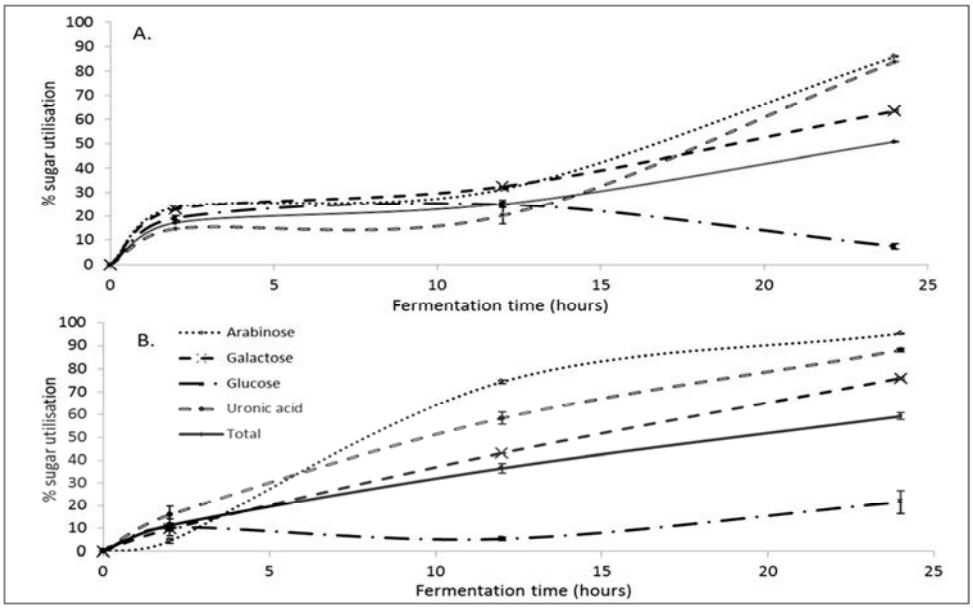

Figure 3-4. Utilization (%) of monosaccharides during in vitro fermentation originally present in **A.** Chicory root pulp (CRP) and **B.** Ensiled chicory root pulp (ECRP). Values are means of duplicate measurements with errors shown by vertical bars on the curves.

The enzyme machinery from the fermenting bacteria seemed to be so efficient in degrading arabinan that the 14% level of resistant arabinan after 24 h of fermentation is only half of

the arabinan remaining in the cell wall matrix after severe extraction by strong alkali (*11*). This indicated that even part of the heavily entangled arabinan was utilized during fermentation.

Changes in ensiled CRP fiber composition during fermentation

The presence of more soluble fibers in a more open cell wall network in ECRP compared to CRP was hypothesized to have an effect on the fiber utilization during fermentation of the cell wall network. The constituent monosaccharide compositions of the fermentation digests obtained during fermentation of ECRP are shown in table 3.2.

After 24 h of fermentation, 59% of all carbohydrates are utilized from ECRP (figure 3.4B). The 8% higher carbohydrate utilization compared to CRP indicates a slightly higher fermentability of ECRP over CRP as has been seen from the higher SCFA production as discussed earlier. Unexpectedly, the increase in SCFA production (30%) is much higher than the 8% higher carbohydrate utilization. ECRP fermentation was also quicker as was indicated from the high level of carbohydrate utilization (36%) within a short time period of 12 h compared to only 25% carbohydrate utilization for 12 h of CRP fermentation (table 3.2).

As for CRP fermentation, specific CWPs consisting of Ara, GalA and Gal are favourably utilized the most during ECRP fermentation, but to a larger extent compared to CRP fermentation (figure 3.4). As seen in figure 3.4, the sugar utilization for ECRP is more pronounced than CRP fermentation after 12 h than after 24 h of fermentation. At 12 h, the sugar utilization was determined to be (31 and 74%) Ara, (20 and 58%) GalA, and (32 and 43%) Gal for CRP and ECRP respectively (figure 3.4). This rapid increase in carbohydrate utilization coincides with the higher levels of soluble fibers composed of Ara, Gal and GalA in ECRP. This indicated that processing of CRP, increasing the levels of soluble fibers and modifying the cell wall network, increased the degradability and the rate of fermentation of specific CWPs.

Utilization of soluble/insoluble fibers from cell wall materials during fermentation

The presence of soluble fibers and/or more open CWP network of the remaining insoluble fibers in ECRP seem to enhance fermentation. In order to elaborate on this observation, the levels of both soluble and insoluble fibers in digests are shown in figure 3.5.

Utilization of soluble/insoluble fibers from CRP:

The insoluble fibers in CRP are utilized only after 12 h when all soluble fibers have been utilized. This is also in agreement with HPSEC in which no soluble fibers could be seen after 12 h of fermentation (figure 3.2). Insoluble fibers are mostly utilized between 12 and 24 h of fermentation shown by the decrease from 73% to 48% of residual insoluble carbohydrates. Insoluble fiber utilization mostly involved pectic sugars of Ara, GalA and Gal as indicated from table 3.3. Only 18% of all insoluble pectin (GalA+ Rha + Ara + Gal) (calculated from table 3.3) remained unfermentable after 24 h suggesting a high fermentability of insoluble pectin from the CRP plant cell wall.

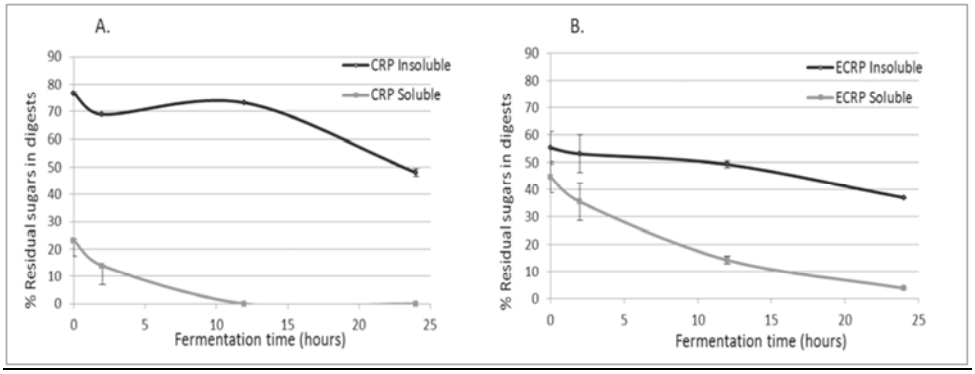

Figure 3-5. Proportion of sugars in soluble and insoluble fractions of (%) original sugars present. **A.** Chicory root pulp (CRP) fermentation and **B.** Ensiled chicory root pulp (ECRP) fermentation. Values are means of duplicate measurements with errors shown by vertical bars on the curves.

For ECRP fermentation, utilization of soluble fibers can be seen from the decrease in the residual soluble carbohydrate levels from 45 to 4% from 0 to 24 h (figure 3.5B). Utilization of insoluble fibers is slow and lower than found for the soluble fiber as shown by the decrease from 55% to 37% of residual insoluble carbohydrates (figure 3.5B). Soluble fibers consisting of pectic sugars (table 3.3) are thus preferentially utilized. This suggests that the

increased rate and higher level (8%) of carbohydrate utilization in ECRP fermentation compared to CRP fermentation is due to fermentation of soluble pectin fibers that are present in high amounts in ECRP.

Table 3-3. Proportion of constituent monosaccharides present in soluble and insoluble fractions of % original carbohydrates during *in vitro* fermentation of chicory root pulp (CRP) and ensiled CRP (ECRP).

	Time (hours)	Rha	Ara	Xyl	Man	Gal	Glc	UA	Total
CRP		Sugars in insoluble fraction							
	0 (blank)	62	81	84	50	72	76	80	77
	2	52	66	70	50	58	69	75	69
	12	91	67	68	46	65	75	79	73
	24	43	14	88	52	35	92	14	48
		Sugars in soluble fraction							
	0 (blank)	38	19	16	50	28	24	20	23
	2	37	11	26	48	20	12	10	14
	12	9	0	12	0	0	0	0	0
	24	15	0	0	0	1	0	2	0
ECRP		Sugars in insoluble fraction							
	0 (blank)	47	39	100	61	55	80	33	55
	2	45	33	96	60	47	79	31	53
	12	37	19	93	60	41	79	28	49
	24	26	5	86	56	16	76	9	37
		Sugars in soluble fraction							
	0 (blank)	53	61	0	39	45	20	67	45
	2	49	62	2	31	43	10	53	35
	12	38	7	3	24	15	16	14	14
	24	35	0	5	8	8	3	3	4

Values are means of duplicate measurements. Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA). Standard errors of the values are presented in table 3.S2 (supplementary data).

Compared to CRP, for ECRP, only 9% of all pectin (Rha+Gala+Ara+Gal) originally present remained unfermentable (calculated from table 3.3). This suggested that pectin in the hypothesized more open structure of the ECRP cell wall was more degradable than the pectin in the CRP cell wall structure contributing to an increased fermentability of ECRP. Other than pectin, the ECRP cell wall contained quite some cellulose + hemicellulose

sugars (glucose+ xylose+mannose) that were unfermentable (75%) (calculated from table 3.3). This suggested that the fermentative degradation of the CWP network was selective for pectin fibers and preferably soluble pectin. This is in agreement with the limited degradation of (hemi)cellulose by the human gut microbiota in human subjects (*28*).

Microbiota composition during CRP fermentation

The microbiota composition during the course of fermentation is expected to be modulated based on the composition of the fiber added during fermentation (*29*). The microbiota composition of the fermentation digest for CRP fermentation (figure 3.6) is expressed as % abundance of genera of the total microbiota in the fermentation digest. The fermentation digest at 24 h of fermentation showed an increase in *Clostridium sensu stricto sp.* (1.34%) and *Lachnospiracea incertae sedis sp.* (1.21%). The highest increase was seen for *Bacteroides sp.* (8%), which remain the most dominant bacteria after 24 h of fermentation. Although *Lachnospiracea incertae sedis sp.* is a butyrate producer (*30*) and increased during fermentation, the observed lower butyrate : (acetate + propionate) ratio of 6:94 after 24 h compared to 50:50 at the start of fermentation (figure 1) could be due to the decrease in butyrate producers (*31*, *32*) mostly from *Clostridium XIVa sp. (*6%*), Faecalibacterium sp.*(0.94%), and *Butyricicoccus sp.* (0.57%). Other bacteria which decreased in 24 hours were *Sutterella sp.* (5.7%), *Dorea sp.* (1.5%) and *Clostridium XI sp.* (1%). *Coprococcus sp.*, *Dialyster sp.* and *Bifidobacterium sp.* remained more or less the same. Despite the presence of the bifidogenic inulin fraction (*33*), no growth of *Bifidobacterium sp.* was seen after 24 h of fermentation, since inulin (6% of all CRP carbohydrates) was already utilized within 6 h of fermentation. Although changes in the microbiota composition are seen during CRP fermentation, the significance of the change in the complex community of microbiota is hard to understand.

Part of the significance may be understood when correlated to metabolite production, fiber degradation and utilization. It is likely that *Bacteroides sp*., identified as the dominant bacteria in fermenting CRP, are responsible for the shown utilization of arabinan, galactan and homogalacturonan and an increase in acetate and propionate. *B.thetaiotaomicron* known to be highly abundant among *Bacteroides sp* (*34*). has been estimated to include 236

glycoside hydrolase genes (*35*, *36*). Production of enzymes during the course of fermentation is upregulated based on the polysaccharides present during fermentation (*35*).

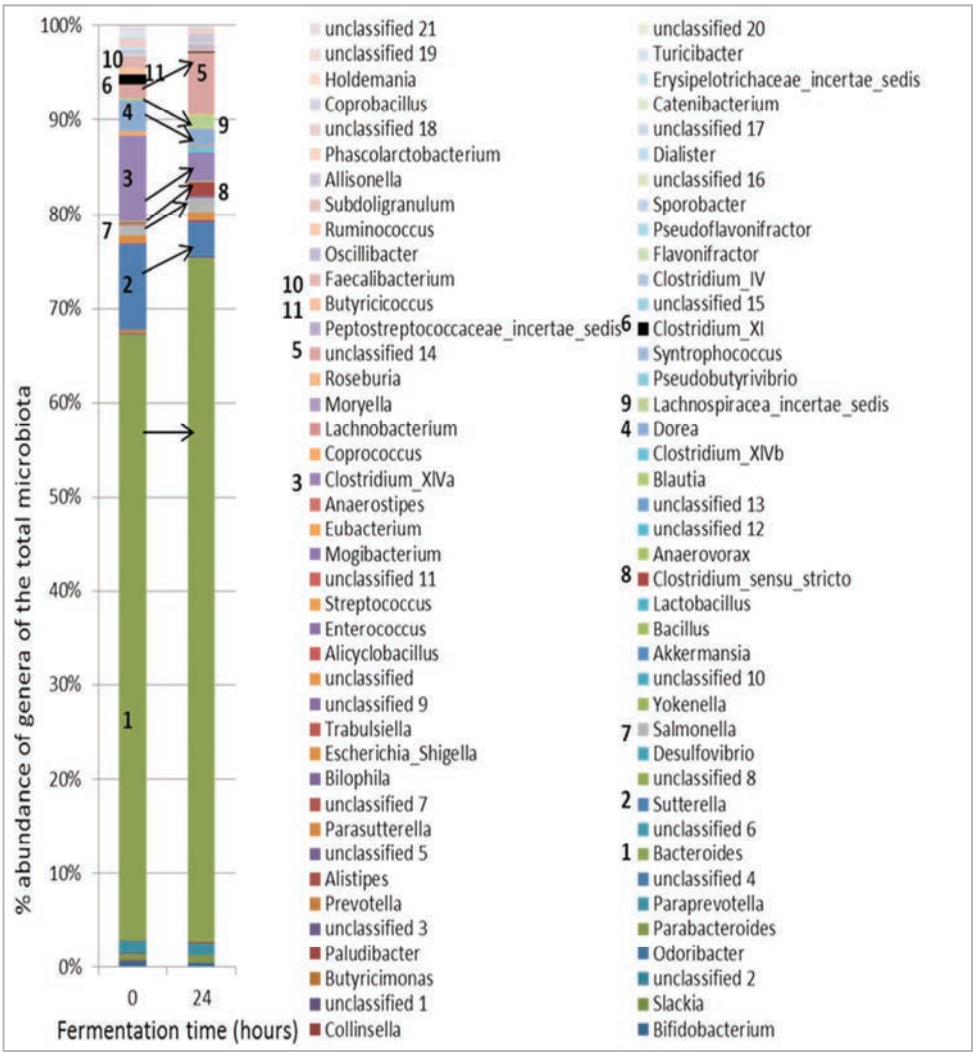

Figure 3-6. Microbiota composition during fermentation of chicory root pulp (CRP) expressed as % abundance of genera of the total microbiota in the fermentation digest. Numbers in the legend indicate operation taxonomic units (OTUs) which are highlighted in the graph

3.4. Conclusions

This study revealed that processing (ensiling) of chicory root pulp to open the cell wall network and solubilize pectin from the cell wall network will increase the fermentation rate of soluble pectin fibers as well as insoluble pectin fibers in the pulp. Processing an agricultural by-product would thus be an option if an increased fermentation rate of cell wall polysaccharides is desired for the final dietary fiber product.

3.5. Acknowledgements

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Supplementary data:

Table 3-S1. Values representing standard deviations for short chain fatty acids (SCFA's) and organic acids measured in duplicates during fermentation of chicory root pulp (CRP) and ensiled CRP (ECRP)

CRP	Acetate	Propionate	Butyrate	Total SCFA	Lactate	Succinate
0i	0.01	0.03	0.63	0.59	0.00	0.05
0	0.25	0.42	1.54	2.21	0.01	0.08
2	0.05	0.02	0.19	0.27	0.03	0.15
6	0.24	0.03	0.04	0.16	0.03	0.18
8	0.81	0.68	0.88	2.37	0.04	0.12
12	2.17	0.97	0.92	0.28	0.01	0.06
24	0.26	0.32	0.86	0.92	0.00	0.02
ECRP	Acetate	Propionate	Butyrate	Total SCFA	Lactate	Succinate
0i	0.15	0.16	0.39	0.71	0.00	0.01
0	0.15	0.31	0.85	0.40	0.00	0.00
2	0.06	0.02	0.40	0.33	0.00	0.01
6	0.32	0.03	0.37	0.03	0.01	0.01
8	0.27	0.07	1.22	1.42	0.02	0.00
12	0.88	0.34	0.42	1.63	0.00	0.00
24	1.17	0.73	1.21	0.77	0.00	0.01

Table 3-S2. Values represent standard error of means of values presented in table 3.3.

	Time (hours)	Rha	Ara	Xyl	Man	Gal	Glc	UA	Total
CRP					Sugars in insoluble fraction				
	0 (blank)	1.5	0.4	0.7	0.7	0.3	0.5	0.5	0.5
	2	2.7	0.0	0.4	1.6	0.4	0.4	0.6	0.3
	12	0.0	1.7	0.4	2.2	1.0	0.2	0.6	0.3
	24	0.6	0.0	3.2	0.3	2.7	0.8	1.8	1.3
					Sugars in soluble fraction				
	0 (blank)	1.5	0.4	0.7	0.7	0.3	0.5	0.5	0.5
	2	2.7	0.0	0.4	1.6	0.4	0.4	0.6	0.3
	12	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0
	24	0.6	0.0	0.0	0.0	2.7	0.0	1.8	0.0
ECRP					Sugars in insoluble fraction				
	0 (blank)	5.0	2.2	0.0	6.0	3.8	8.1	4.3	5.8
	2	1.9	2.2	16.9	10.3	5.0	13.1	1.8	6.9
	12	0.1	0.1	5.8	1.8	1.5	2.6	0.1	1.3
	24	3.6	0.0	3.2	0.6	0.2	0.7	0.1	0.3
					Sugars in soluble fraction				
	0 (blank)	5.0	2.2	0.0	6.0	3.8	8.1	4.3	5.8
	2	1.9	2.2	16.9	10.3	5.0	13.1	1.8	6.9
	12	0.1	0.1	0.0	1.8	1.5	2.6	0.1	1.3
	24	3.6	0.0	3.2	0.6	0.2	0.7	0.1	0.3

Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

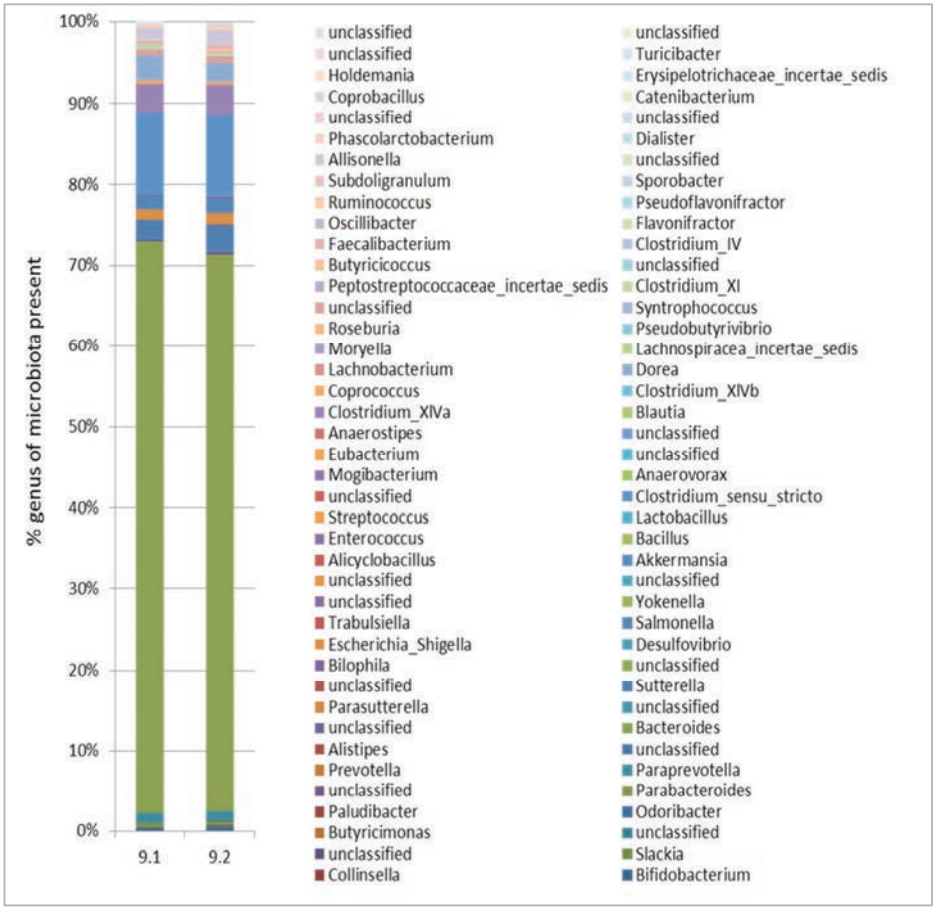

Figure 3-S1. Microbiota composition of independent duplicates of a 9 h fermentation of chicory root pulp (CRP) expressed as % operation taxonomic units (OTUs) of microbiota present

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Chapter 4

The fate of chicory root pulp polysaccharides during fermentation in the TNO *In vitro* model of the colon (TIM-2)

Submitted for publication

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Abstract

The aim of this study was to monitor cell wall polysaccharide (CWPs) utilization during fermentation by the human colonic microbiota in the TNO *In vitro* model of the colon (TIM-2). Chicory root pulp (CRP) and treated (ensiled) CRP (ECRP) containing four times more soluble pectin than CRP, were fermented in the model. After the adaptation phase of the human faecal inoculum to CRP and ECRP for 48 h, both materials were fermented quite rapidly (57% carbohydrate utilization in 2 h). ECRP carbohydrates (85%) were less fermented in 24 h compared to CRP carbohydrates (92%). It was hypothesized that soluble fibers that are readily fermentable and dominantly present in ECRP programmed the microbiota in TIM-2 to fully adapt to these soluble fibers. The microbiota was not able to change towards the fermentation of insoluble fibers anymore. Consequently, ECRP insoluble fibers were less utilized than CRP insoluble fibers in TIM-2 leading to an overall lower fiber utilization and SCFA production.

4.1. Introduction

Dietary fiber (DF) representing cell wall polysaccharides (CWPs), such as pectin, hemicellulose and cellulose are resistant to digestion in the human small intestine and become available for (partial) fermentation in the colon by the human microbiota (*1*). Numerous models have been used to study fiber fermentation in the colon. Compared to *in vivo* fermentation studies, *in vitro* fermentation models can be used without ethical constraints and they enable monitoring of the degradation of fiber through time. In addition, *in vitro* fermentation experiments are more simple to perform than *in vivo* fermentation experiments (*2*) and there is no interference with other food components in the investigation of the effect of fibers themselves (*3*).

In vitro gut fermentation models range from simple closed system models (batch models) to complex continuous flow (dynamic) models (*4*). Fermentation in a batch model is simple, inexpensive and high throughput. The fermentation is done in a highly buffered system in order to have a stable pH due to the accumulation of the main fermentation metabolites short chain fatty acids (SCFA). In contrast, in continuous gut fermentation models, such as the TNO *In vitro* Model of the colon (TIM-2) (*5*), the pH can be controlled and the fermentation metabolites are not accumulated. Instead these metabolites are partly removed through dialysis thus resulting in a stable physiological pH that mimics the human proximal colon. Due to the removal, continuous gut fermentation models have the capacity to handle large densities of bacteria as found in the colon. These features make them closer to simulating the human colon than batch models (*4*).

The TIM-2 has been designed to simulate conditions of the lumen in the human proximal colon. The lumen functions to provide peristaltic mixing of components while water and fermentation metabolites are absorbed via a hollow fiber membrane running through the lumen (*5*). The TIM-2 system has been used in testing the fermentability of fibers based on the analysis of fermentation metabolites, such as SCFA, and changes in the microbiota composition (*6, 7*). However, fiber utilization and changes in the CWP composition during fermentation in TIM 2 were not studied so far.

Fiber rich agricultural by-products, such as chicory root pulp (CRP) and ensiled chicory root pulp (ECRP), are abundant in CWPs (56-58 w/w%) (*8*). ECRP has a modified cell wall

network and a higher ratio of soluble: insoluble fibers compared to CRP (*8*). After fermentation of CRP and ECRP, in a batch model for 24 h, it was demonstrated that 51% of the CRP carbohydrates were readily utilized. The higher levels of soluble fiber and the hypothesized more open network in ECRP increased the rate of fiber utilization by another 11% in 12 h compared to CRP. The overall extent of utilization was higher by 8% for 24 h compared to CRP. (*9*).

Fermentation of CRP and ECRP may be different in TIM-2. Along with peristaltic movement and dialysis of metabolites (*5*), TIM-2 is inoculated with a microbiota with the same microbial density as found *in vivo (5)*. This is atleast 10 fold higher than our previous batch experiments (*9*). This may have an influence on the extent and the rate of fermentation of both cell wall materials. Compared to the batch, the microbiota in TIM-2 are also adapted to the cell wall material which may an influence on the fermentation of the cell wall material.

This study was thus aimed at determining and understanding the fate of CWPs from both untreated and treated chicory root pulp during fermentation in the TIM-2. Next to the formation of SCFA, the solubilisation and utilization of individual sugars present in the cell wall in time was monitored.

4.2. Materials and Methods

CRP and ensiled CRP were obtained from Cosun Food Technology Center (Roosendaal, The Netherlands) and have been described previously (*8*). Since both materials mainly contain dietary fibers and no starch and digestible oligosaccharides that would be digested in the stomach or the small intestine, no pre-digestion was performed on these pulp materials.

In vitro fermentation in TIM-2

The model set up of TIM-2 is as described before (*6*). Briefly, conditions in the proximal colon (pH 5.8, anaerobe conditions, removal of microbial metabolites) were mimicked. The pH was controlled by addition of sodium hydroxide (NaOH). The fermentation medium used in TIM-2 was modified standard ileal efflux medium (SIEM), which simulates the material entering the colon (*6*). The components in SIEM, as described earlier (*6*) included

(g/L): 9.4 pectin, 9.4 xylan, 9.4 arabinogalactan, 9.4 amylopectin, 47 casein, 78.4 starch, 47 bactopeptone and 0.8 oxbile with the exception that Tween 80 was omitted from SIEM. The latter was found to hinder further analysis of sugar degradation products (data not shown).The composition of dialysate is as described before (*6*).

The inoculum used in TIM-2 was a standardized microbiota prepared from the fecal material from eight healthy European adults (25-45 years old) who did not receive antibiotics in 2 months before donation (*6*).

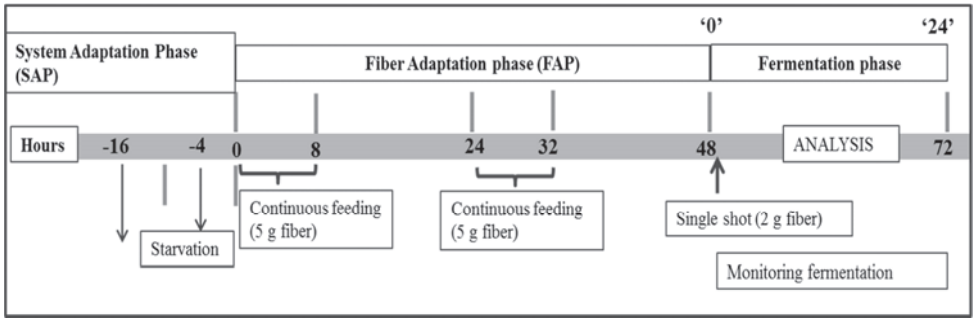

Figure 4-1. Schematic presentation of experimental set-up for fiber addition in the TNO *In vitro* Model of the colon (TIM-2)

Figure 4.1 shows the experimental set-up for fiber addition to the model. The microbiota was allowed to adapt to the model and SIEM for 16 h denoted as 'system adaptation phase' (SAP). The SIEM added contained the complex carbohydrates as mentioned above. The SIEM carbohydrates were added at a rate of 2.5 mL/h. At the end of SAP, a 4 h starvation period was incorporated where all available carbohydrates in the medium were allowed to be fermented by the microbiota which was indicated from a constant pH without further NaOH addition (*5*). Then the microbiota was allowed to adapt to the test substrate in the model for 48 h in two slots of 24 h each, denoted as 'fiber adaptation phase' (FAP). In each slot, carbohydrates in SIEM were replaced with 5 g of the fiber which was added continuously in the model (0.625 g in 2.5 mL SIEM /h) during the first 8 h. This was then followed by the 'fermentation phase' (figure 4.1) where a single shot of 2 g test substrate was added to the model and the incubation was continued for 24 h. As shown in figure 4.1, '0' is equivalent to '*the time just before fiber addition'* for fermentation to start in the experimental fermentation phase.

After fiber addition to the model, no sample was withdrawn from the model for analysis. This was done to allow sufficient time for homogenous mixing of the fiber components in the model through peristaltic movements so as to enable mass balance analysis of carbohydrates during fermentation.

Samples (4 mL) were taken from the lumen after 0 h (prior to fiber addition) and after 48 h of FAP and at 15 min, 30 min, 2, 4, 8 and 24 hours of the fermentation phase for monitoring fiber degradation and utilization and for SCFA analysis. Samples from the dialysate were taken after 0, 24 and 48 h of FAP and at 8 and 24 h of the fermentation phase for SCFA analysis.

Fermentation digests were inactivated at 100°C for 5 min and split into two parts. One part was reserved for direct analysis and the other part of the fermentation digest was freeze dried for further characterization.

Part of the dried fermentation digest was suspended in millipore water (10mg/mL) at room temperature and centrifuged (10 min, 9,000 x g, 4°C) to obtain soluble and insoluble fractions. The total fermentation digest and the insoluble fraction (freeze dried) were analysed for constituent monosaccharide content and composition. Constituent monosaccharide content of the soluble fraction was calculated from the difference in individual monosaccharide contents between the total fermentation digest and the insoluble fraction of the fermentation digest.

Analytical methods

Constituent monosaccharide composition

The total constituent monosaccharide content and composition were determined using as described elsewhere (*8*). Measurements were performed in duplicate. Overall, the coefficient of variation for the measurement of the sugar composition was below 8%.

Oligosaccharide profiling

High Performance Anion Exchange Chromatography (HPAEC) was performed on a ICS5000 system (Dionex, Sunnyvale, CA, USA), equipped with a pulsed amperometric detection and Dionex CarboPac PA-1 column (2 x 250 mm) in combination with a Carbopac PA-1 guard column (2 x 50 mm) as described elsewhere (*3*). The soluble fraction

of the fermentation digests were obtained by centrifugation (10min, 18,000 x g, 24°C). Ten μl of the soluble fraction (diluted twice) was injected into the system.

Analysis of Short Chain Fatty Acids (SCFA)

Short Chain Fatty Acids (SCFA) present in digests and dialysates were analysed as described by Maathuis et al (*6*).

4.3. Results and discussion

Cell wall materials used for fermentation in TIM-2

Chicory root pulp (CRP) and ensiled CRP (ECRP) described earlier (*8*) are abundant in CWPs (56-58 w/w (%)) and have rather similar sugar compositions (table 4.1). In addition, CRP contains 6 w/w (%) inulin which is not present in ECRP due to the ensilage process. The CWPs in CRP and ECRP calculated from the mol% monosaccharide composition and total sugar content (w/w (%)) (table 4.1) for both materials consist of 62 % pectin (uronic acid + rhamnose + arabinose + galactose), 11% hemicellulose (mannose + fucose + xylose + glucose in xyloglucan, based on the ratio of xylose:glucose (3:4) in CRP/ECRP xyloglucan) and 27% cellulose (arising from glucose not present in xyloglucan) as characterised earlier (*8*). The carbohydrates present in the soluble fraction from CRP represent only 12% of the total carbohydrates, half of which is arabinose rich pectin and the other half is inulin. 87% of the carbohydrates from CRP are insoluble and exhibit a similar polysaccharide composition as the total CRP (table 4.1). The ECRP does not contain inulin (*8*), but contains four times more soluble fibers in the soluble fraction (48%) compared to CRP (12%) (table 4.1) (*8*). As indicated from the sugar composition, these soluble fibers are abundant in pectic sugars. As a consequence of a higher carbohydrate recovery in the soluble fraction, the insoluble fraction represents less carbohydrates (55%) of ECRP carbohydrates compared to CRP (87%) (table 4.1).

Table 4-1. Constituent monosaccharide composition of chicory root pulp (CRP) and ensiled chicory root pulp (ECRP)

	Mol %								(w/w%)	Carbohydrate yield [g in fraction per100g in CRP/ECRP]
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	Total	
CRP	1	0	15	4	4	7	31	38	64*	100
CRP Soluble	1	0	27	1	13	6	19	33	50	12
CRP Insoluble	1	0	12	4	2	7	33	40	68	87
ECRP	2	0	15	4	3	7	31	38	56	100
ECRP Soluble	2	0	27	0	3	8	3	57	62	48
ECRP Insoluble	1	0	5	7	3	5	56	21	55	55

Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA), * contains 6 w/w % inulin. Results from an earlier study (8).

Fermentation of cell wall materials in TIM-2 identified from SCFA levels

The model fermented CRP and ECRP well as indicated from increase in levels of short chain fatty acids (SCFAs) produced and utilization of fibers (discussed later).

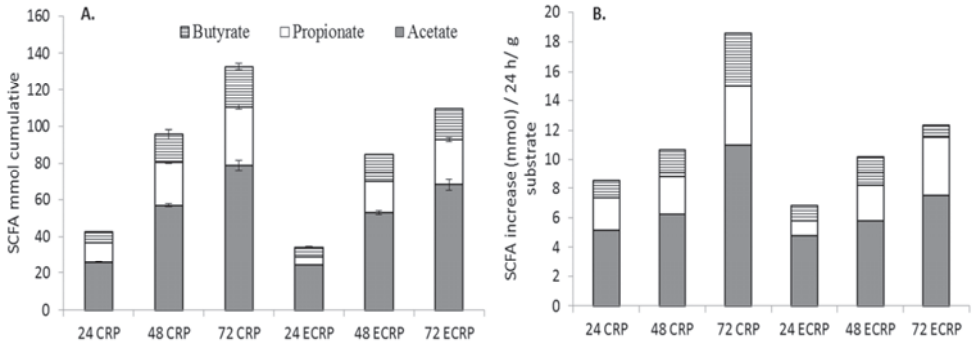

Figure 4-2. Short Chain Fatty Acid (SCFAs) cumulative (mmol) shown in **A.** and SCFA increase/ g substrate/ 24 h shown in **B.** for chicory root pulp (CRP) and ensiled chicory root pulp (ECRP) in the TNO *In vitro* Model of the colon (TIM-2).

Cumulative SCFA (mmol) produced by the microbiota in TIM-2 are shown in figure 4.2A. Since the substrate is fed in the model in three distinct 24 h time slots, an increase in SCFA levels for each 24 h slot was calculated per g substrate and plotted in time (figure 4.2B).

SCFA levels and microbiota composition during CRP fermentation:

The first 24 h of 'fiber adaptation phase' (FAP) resulted in an increase of 8 mmol SCFA/g substrate (figure 4.2A). A similar increase in SCFA levels were observed during fermentation of CRP in a batch model (7 mmol/g) after 24 h (*9*) despite the fact that the density of the microbiota in TIM-2 was higher compared to the batch model.

Analysis of the microbiota composition for the sample taken before FAP showed a dominant presence of facultative anaerobes (e.g. *Enterococcus*) (data not shown). This indicated that either during the production of the standard inoculum these facultative anaerobes thrived and dominated the final mixture or that during the 'system adaptation phase (SAP)' the microbiota shifted in composition. The latter has been observed before (*10, 11*). The digest obtained after 24 h of fermentation phase showed an abundance of *Bacteroides* and bacteria from the Lachnospiraceae family (data not shown). It is believed that the increase in these bacteria in TIM-2 already occurred during the first FAP since a constant ratio of acetate: propionate: butyrate (58:23:18) was seen throughout FAP and the fermentation phase. We have observed on many occasions that shifts in activity in TIM-2 occur swiftly and are maintained throughout the experimental period e.g.(*12*). Compared to the TIM-2, the microbiota composition was dominanted by *Bacteroides* after 24 h of CRP batch fermentation (*9*). As a consequence, the ratio of acetate: propionate: butyrate (77:16:6) for the batch fermentation is also different compared to the TIM-2 fermentation (58:23:18) which has higher propionate and butyrate levels. The difference in the microbiota composition is obvious to arise from the different set-up of the two fermentation models.

For the second time slot of 24 h in TIM-2, an increase by 11 mmol SCFA/g was seen.

After the final addition of a single shot of 2 g CRP in the model and incubation for another 24 h, a higher increase in SCFA, 15mmol/g was seen pointing to an increased fermentability of CRP with repeated addition of CRP. Due to the adaptation of bacteria to CRP in the model for 2 days and increase in numbers of those bacteria that are able to

ferment CRP, the fermentability of CRP with the addition of only a single shot of CRP resulted in a higher conversion of fiber into SCFAs. An increase in fermentability was also observed for the soluble fiber, pullulan fed to TIM-2 (*6*). However, the calculated increase in SCFA levels for pullulan from FAP to fermentation phase (8 mmol/g) is lower compared to CRP. This may be due to the dominant presence of bifidobacteria found for pullulan (*6*) compared to *Bacteroides* and bacteria from the Lachnospiraceae family found for CRP fermentation.

SCFA levels during ECRP fermentation

As seen for CRP, adaptation of ECRP was also shown from increased fermentabilities of fiber in the FAP and fermentation phase (7, 10 and 12 mmol/ 24 h/ g substrate) respectively (figure 4.2B). These levels were lower than CRP (figure 4.2B) especially after the fermentation phase. This suggested that the higher fermentability of CRP over ECRP was due to a different adaptation of bacteria towards CRP. Unlike batch fermentation which showed a 30% higher SCFA production for ECRP fermentation than for CRP fermentation, the TIM-2 fermentation of ECRP and CRP implied that ECRP containing more soluble fibers and having a hypothesized more open cell wall network than CRP was less fermentable than CRP. This may arise from differences in microbial populations specifically degrading soluble or insoluble fibers in CRP and ECRP TIM-2 fermentations. The lower overall fiber fermentation of ECRP compared to CRP is in line with the slightly lower SCFA production compared to CRP.

The TIM-2 adaptation

The levels of constituent monosaccharides after the system adaptation phase (SAP) (0 h) and fiber adaptation phase (FAP) (48 h) for CRP and ECRP are shown in table 4.2.

Carbohydrates after SAP

The time = 0 h in the model was set after 4 h of starvation after SAP (as shown in figure 4.1), based on constant pH achieved (no NaOH added to control pH) to perceive complete fermentation of carbohydrates in the model (*5*). This ‘complete fermentation’ of carbohydrates does not necessarily indicate complete utilization of SIEM carbohydrates. For this reason, fiber levels were analysed at 0 h. As seen from table 4.2, the 0 h digest

contains carbohydrates but in low levels (146 mg in total present in the TIM-2 system) representing 3% of carbohydrates from SIEM that were added during SAP.

Table 4-2. Levels of constituent monosaccharides before and after addition of chicory root pulp (CRP) and ensiled CRP (ECRP) during TIM-2 fermentation

	Carbohydrate amount	mg in TIM-2						
	mg in TIM-2	**Rha**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**	**UA**
CRP fermentation								
After SAP	146	9	5	20	1	42	46	23
10 g CRP addition	6440	80	780	200	240	450	1990	2690
After FAP (48 h)	321	22	8	54	14	33	155	33
% in FAP from CRP	5	28	1	27	6	7	8	1
After CRP addition	1609	38	164	94	62	123	553	571
% Carbohydrates from FAP	20	58	5	57	23	27	28	6
ECRP fermentation								
After SAP	262	15	6	30	2	59	116	33
10 g ECRP addition	5570	70	680	190	170	380	1710	2360
After FAP (48 h)	434	28	7	62	15	42	193	86
% in FAP from ECRP	8	41	1	33	9	11	11	4
After ECRP addition	1548	42	143	100	49	118	535	558
% Carbohydrates from FAP	28	67	5	62	31	35	36	15

Rhamnose (Rha), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA), SAP (system adaptation phase), FAP (fiber adaptation phase).

Glucose (Glc) present (46 mg) may arise from resistant starch and amylopectin as well as from bacterial cell walls. However it has been shown that during Glc fermentation in TIM-2 that SCFA represented 91% of the ^{13}C carbon balance indicating that bacteria do not contribute much to Glc remaining in the model (*13*). The Glc left after SAP however represents only 1% of all Glc fed to the system as starch and amylopectin indicating that these fibers were well fermented in SAP. Galactose (Gal) (42 mg; 11%), xylose (Xyl; 5%)

(20 mg) and galacturonic acid (GalA) (23 mg; 6%) that remained in TIM-2 after SAP originated from arabinogalactan, xylan and pectin in SIEM respectively.

Carbohydrates after FAP:

After fermentation of 10 g CRP in FAP for 48 h, 5% of CRP carbohydrates remained in the model (table 4.2). Glc levels in the model represented 8% of CRP Glc from xyloglucan and cellulose. Other remaining sugars (table 4.2) are believed to arise from CRP pectin and mannan. When continuing with the experimental fermentation phase, after 2 g addition of CRP, the carbohydrates in TIM-2 included carbohydrates from CRP and carbohydrates that remained from FAP (table 4.2). Thus carbohydrate levels monitored during the fermentation phase included carbohydrates arising from CRP (80%) and carbohydrates that remained after FAP (20%).

As found for CRP fermentation, carbohydrates also remained after FAP of ECRP (table 4.2). Compared to the 48 h CRP digest, 100 mg of ECRP carbohydrates remained. This was not fully expected since ECRP is considered to be fermented more easily than CRP as was seen from batch fermentation (*14*) (*9*). The carbohydrates after FAP represented minor proportion (8%) of ECRP carbohydrates added to TIM-2 (table 4.2). Thus carbohydrates present in the fermentation phase include carbohydrates arising from ECRP (72%) and carbohydrates that remained after FAP (28%).

Carbohydrate utilization in TIM-2 during the CRP fermentation phase

Utilization of fibers from CRP during fermentation was indicated from the decrease in fiber levels in time. The total carbohydrate content of all the fermentation digests (table 4.3) was low due to the presence of other media components, such as proteins and salts. Carbohydrate utilization in the model is very rapid as shown by 57% carbohydrate utilization within 2 h. The level of utilization strongly increased during the first 4 h where 78% of all carbohydrates are utilized (figure 4.3A). To define which polysaccharides are utilized most quickly and intensely during fermentation, the utilization in time of the dominant CRP sugars (arabinose (Ara), Gal, GalA and Glc) is shown in Figure 4.3A.

Table 4-3. Constituent monosaccharide composition during TIM-2 fermentation of chicory root pulp (CRP)

Fermentation time	Carbohydrate amount	Mol %							(w/w%)
(hours)	(mg in TIM-2)	Rha	Ara	Xyl	Man	Gal	Glc	UA	Total
FAP 0	146	7	4	16	0	28	30	14	7
FAP 48	321	7	3	20	4	10	46	9	7
After fiber addition	1609	3	12	7	4	8	34	32	26
0.25	1104	3	10	9	3	8	43	24	22
0.5	932	3	8	10	3	8	45	22	19
2	680	4	5	13	4	7	47	20	15
4	339	6	3	15	4	9	44	18	8
8	239	6	2	22	4	9	41	16	7
24	122	11	4	20	5	14	29	15	4

Rhamnose (Rha), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

Specific carbohydrate utilization in 4 h during CRP fermentation

Rapid utilization of Ara (95%) and GalA (88%) was seen in 4 h (figure 4.3A). In addition, Gal (74%) and glucan from (hemi)cellulose (72% Glc) (figure 4.3A) were rapidly utilized. Other (hemi)cellulosic sugars present in minor amounts were also utilized in 4 h (calculated from the mol% sugar composition and total carbohydrate levels/100 mL shown in table 3a) (Xyl (54%) and mannose (Man) (80%)). In contrast, during batch fermentation of CRP, (hemi)cellulose was barely utilized after 24 h (11%)(*9*). This suggests that adaptation of microbiota to CRP increased the utilization of cellulose. The same effect has been reported for beet pulp batch fermented *in vitro* after 3 days of adaptation (*15*) with a high rate of fermentability (37%/h from 3 to 9 h) achieved with a slightly lower ratio of microbiota to substrate (1.4:1) compared to TIM-2 (1.8:1).

Slow polysaccharide utilization from 4 to 24 h during CRP fermentation

From 4 to 24 h, the extent of sugar utilization was slow since 13% of all total carbohydrates were utilized in 20 h (figure 4.3A). Pectic sugars of Ara (4%), GalA (8%) and Gal (12%) were utilized slowly since they were already utilized within 4 h. As a result other sugars

arising from (hemi)cellulose such as, Xyl (23%) and Glc (21%) that were not utilized earlier were utilized in this period of 20 h. This slow fermentation of residual cell wall material is due to low activity of bacteria.

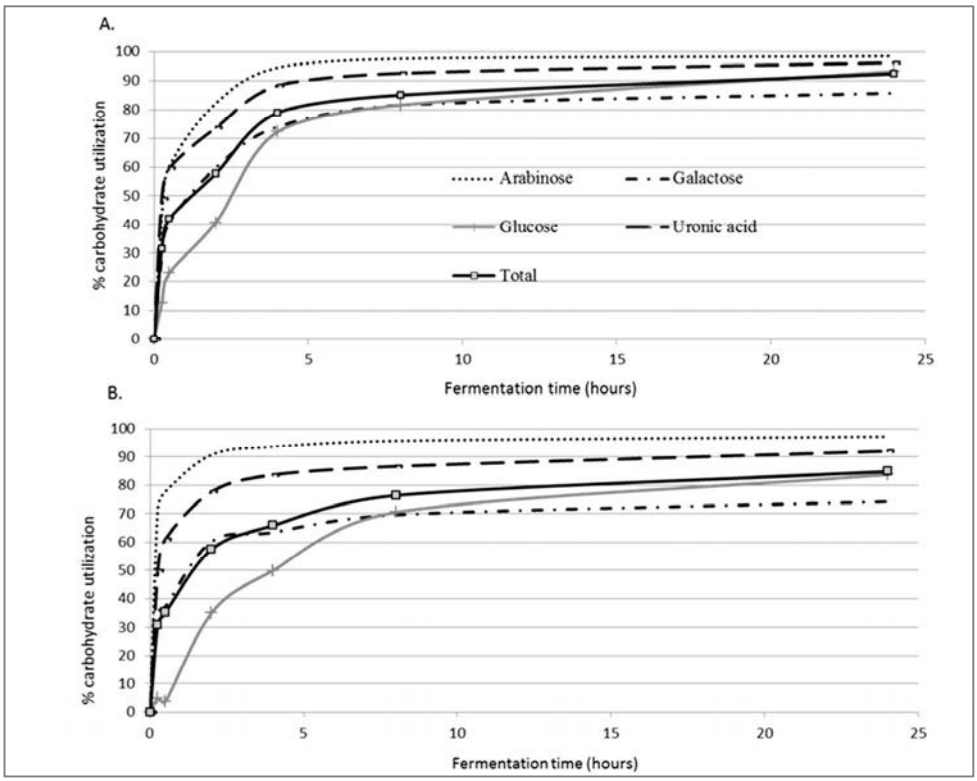

Figure 4-3. TIM-2 fermentation (fermentation phase): Utilization of carbohydrates as % of originally present in **A.** chicory root pulp (CRP) and **B.** ensiled chicory root pulp (ECRP)

Carbohydrate utilization after 24 h of CRP fermentation

After 24 h, 92% of all carbohydrates were utilized (figure 4.3A (solid line)). This indicated a high fermentability of CRP compared to the *in vitro* fermentability of CRP in a batch model (51% disappearance of carbohydrates)(*8*). Ara (99%) and GalA (96%) were almost completely utilized (figure 4.3A).The high fermentability as also revealed from SCFA analysis is due to adaptation of microbiota in the model to CRP. *In vitro* fermentability of another pectin rich material, sugar beet pulp, after adaptation for three days also showed a high fermentability (91%) (*15*).

Fiber utilization in TIM-2 during fermentation of ECRP

The sugar compositions of the ECRP fermentation digests are shown in table 4.4. It can be calculated that after 24 h, 85% of all carbohydrates were utilized indicating high fermentability of ECRP in TIM-2. As seen for CRP fermentation, ECRP fermentation is also rapid (66% carbohydrate utilization in 4 h) (figure 4.3B). This is also due to adaptation of bacteria to ECRP in TIM-2 as was observed for CRP. But in contrast to CRP fermentation, the final carbohydrate utilization after 24 h from ECRP fermentation is lower. This is in line with the SCFA levels which are also lower for ECRP compared to CRP.

The extent of utilization of ECRP Ara was more rapid than for CRP Ara as was seen from figure 4B. In the first 15 min, 72% of Ara from ECRP was utilized compared to 43% Ara from CRP. However, all other sugars except for Ara were utilized to a lesser extent after 4 h compared to CRP (figure 4.4B) contributing to a lower total carbohydrate utilization compared to CRP. After 24 h of fermentation, Ara (97%) and GalA (92%) were utilized almost completely.

Improved carbohydrate utilization by adapting bacteria

The amount of carbohydrates levels left in the model of the 24 h CRP digest (fermentation phase) was lower by 62% compared to the remaining carbohydrates in the 48 h CRP digest (after FAP) (calculated from table 4.3) suggesting that the adaptation phase led to an increased utilization of carbohydrates that remained after FAP. Furthermore, the 48 h FAP digest showed a higher Glc:Xyl (46:20) ratio compared to the 24 h CRP digest (fermentation phase) (29:20) (table 4.3) suggesting that cellulose was more efficiently utilized during the fermentation phase. *Bacteroides* and bacteria from the Lachnospiraceae family dominant in the fermentation phase (data not shown) are capable of utilising cellulose *(16-18)* which explains cellulose utilization in the system. Their capability increased due to adaptation of bacteria to cellulose. For ECRP fermentation, FAP also increased carbohydrate utilization since the 48 h FAP digest had 45% lower carbohydrate levels compared to the 24 h ECRP digest (fermentation phase) (table 4.4).

Table 4-4. Constituent monosaccharide composition during TIM-2 fermentation of ensiled chicory root pulp (ECRP)

Fermentation time	Carbohydrate amount	Mol %							(w/w%)
(hours)	(mg in TIM-2)	Rha	Ara	Xyl	Man	Gal	Glc	UA	Total
FAP 0	262	6	3	14	1	22	43	11	11
FAP 48	434	7	2	17	3	9	43	18	9
After fiber addition	1548	3	11	8	3	8	34	33	23
0.25	1069	3	5	10	3	7	47	24	19
0.5	1005	4	4	11	3	7	50	20	18
2	659	4	2	14	4	7	52	17	14
4	529	6	2	15	4	8	49	16	12
8	366	7	2	17	4	10	42	18	9
24	236	11	2	18	4	12	36	17	6

Rhamnose (Rha), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

Formation of oligomers during CRP and ECRP fermentation

Fermentation degradation products such as oligosaccharides and monosaccharides may be formed during fermentation. Soluble fractions from the fermentation digests of CRP and ECRP were thus analysed for these degradation products.

CRP fermentation

For CRP fermentation (figure 4.4A), almost no mono- and oligosaccharides were present before the fiber was added as shown from 48 h digest after FAP.

The digest does contain minor levels of monomeric GalA (representing only 0.05% of total GalA from CRP) indicating almost complete utilization of monomeric GalA levels in during FAP. Following CRP addition in the model, monomeric GalA levels were increased clearly within 15 min (figure 4.4A). However, while 50 % total GalA utilization in 15 min, the monomeric GalA formed represented only 1.5 % of total GalA present in the digest. This suggested that GalA was utilized almost as rapidly as the formation of monomeric GalA. The same was seen for oligomeric degradation products of GalA and monomeric Ara

and Gal. Absence of these peaks between 2 to 8 h, suggested that the formation of degradation products were formed slowly to be utilized immediately by competing bacteria.

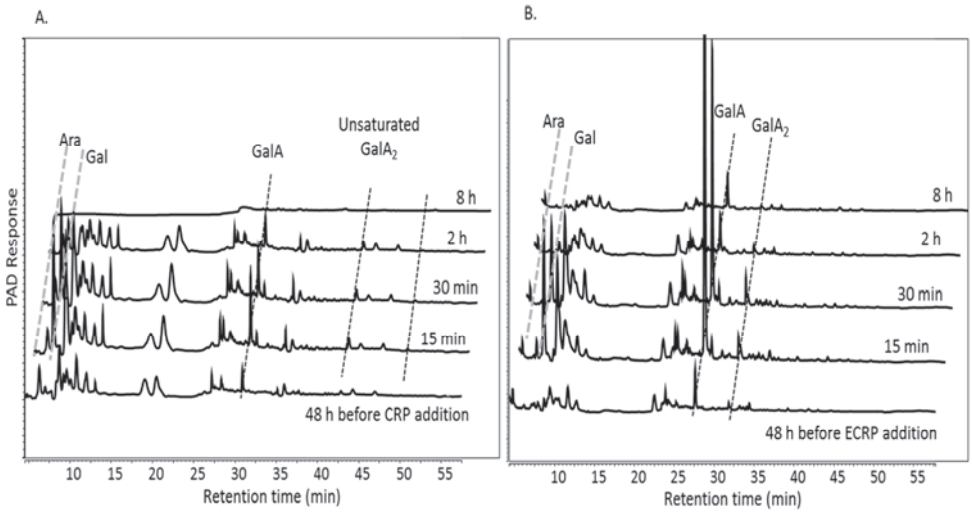

Figure 4-4. HPAEC elution profile of digests obtained during TIM-2 fermentation phase of **A.** chicory root pulp (CRP) and **B.** ensiled chicory root pulp (ECRP)

This is also in line with the slow carbohydrate utilization shown from 4 – 24 h of fermentation (figure 4.3A). The mono- and oligomeric degradation products were not seen during CRP batch fermentation (*9*). This is due to the relatively slow fermentation in a batch model in which degradation products were formed slowly but immediately utilized by bacteria. Compared to CWPS, inulin in CRP is highly fermentable (*19*) and thus was not seen after 15 min of fermentation indicating very rapid utilization of the fiber after adaptation. In a batch fermentation, inulin was utilized within 4 h (*19*). TIM-2 fermentation is thus very rapid where CRP carbohydrates are more completely utilized compared to the batch fermentation.

ECRP fermentation

As seen for CRP fermentation, the model contained minor levels of monomeric GalA in the 48 h digest obtained after FAP (representing only 0.1% of total GalA from CRP), indicating utilization of almost all monomeric GalA during FAP (figure 4.4B).

Within 15 min of ECRP addition in the model, pectin was rapidly degraded to monomeric GalA and oligomers of GalA as was seen for CRP fermentation. But, compared to CRP fermentation, with 50% GalA utilization for CRP and ECRP fermentation, eight times more monomeric GalA was present (12.5% of GalA in the digest). This suggested that the utilization of released monomeric GalA by bacteria was lower in ECRP fermentation compared to CRP fermentation. The same was seen for GalA oligomers and Ara and Gal.

Soluble versus Insoluble fiber utilization from CRP and ECRP

Since the fermentation of cell wall materials in the model was rapid and extensive, the influence of soluble and insoluble fibers on total fiber utilization was further studied. Figure 4.5A and 4.5B show levels of insoluble and soluble fibers in time for CRP and ECRP fermentations respectively.

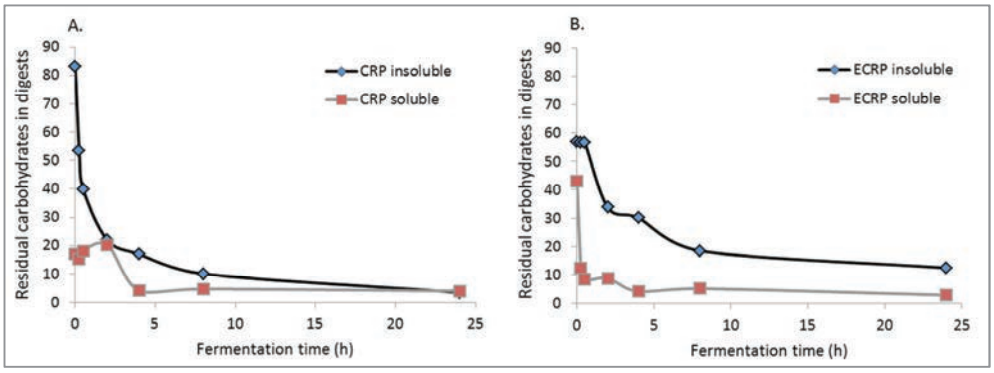

Figure 4-5. Carbohydrates in soluble and insoluble fractions from % original carbohydrates during **A.** chicory root pulp (CRP) fermentation phase and **B.** ensiled chicory root pulp (ECRP) fermentation phase

Carbohydrates in soluble and insoluble fractions in CRP fermentation digests

Percent of sugars as present in either the soluble or insoluble fractions of fermentation digests from CRP are shown in table 4.5. The soluble : insoluble fiber ratio before CRP fermentation was calculated from table 4.5 (83:17) indicating high levels of insoluble fiber in CRP. Levels of insoluble fiber were shown to decrease rapidly by 61 percentage points (pp) until 2 h (figure 4.5A, table 4.5) suggesting that the rapid utilization of fibers from CRP arises mainly due to utilization of insoluble fibers: Ara 72pp, Gal 61pp and GalA 74pp

(table 4.5) were utilized the most. For soluble fibers, an increase in amount was seen until 2 h of fermentation (mainly Rha, Xyl, Man and Glc (table 4.5)) illustrating higher solubilisation of the insoluble fibers than utilization of soluble fibers. Thereafter, a decrease is seen in the soluble fibers within 4 h indicating utilization of soluble fibers as only 4% of total carbohydrates remained. The soluble fiber levels were almost constant until 24 h.

Table 4-5. Sugars present in soluble and insoluble fractions of % original sugars in TIM-2 fermentation digests from chicory root pulp (CRP)

Fermentation digests (h)	Rha	Ara	Xyl	Man	Gal	Glc	UA	Total
	Sugars in insoluble fraction							
Insolubles after CRP addition	81	73	74	58	82	85	88	83
0.25	62	37	87	42	51	72	36	53
2	30	12	38	19	22	30	14	22
4	38	6	45	16	18	22	9	17
24	15	1	9	4	6	3	3	4
	Sugars in soluble fraction							
Solubles after CRP addition	19	27	26	42	18	15	12	17
0.25	28	21	13	13	21	15	15	15
2	32	6	40	20	19	29	12	20
4	17	0	1	4	8	6	3	4
24	21	0	14	6	8	4	1	4
% Disappearance	65	99	77	90	86	93	96	92

Rhamnose (Rha), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

Carbohydrates in soluble and insoluble fractions in ECRP fermentation digests

As expected, carbohydrates before ECRP fermentation have a higher (43:57) soluble: insoluble fiber ratio (table 4.6) than carbohydrates present before CRP fermentation (17:83) (table 4.6). Within 15 min, soluble fibers are utilized rapidly and levels dropped from 43% to 12 % of the total fiber (figure 4.6) indicating a high activity of the fermentation enzymes. In the soluble fraction Ara (75pp), GalA (44pp UA) and Gal (40pp) were utilized the most

within 15 min (table 4.6). The rapid fiber utilization from ECRP thus arises from utilization of the soluble pectin. After 4 h, soluble fibers were almost completely utilized since only 4% of total carbohydrates remained as was seen for CRP fermentation (figure 4.5B).

Table 4-6. Sugars present in soluble and insoluble fraction of % original sugars in TIM-2 fermentation digests from ensiled chicory root pulp (ECRP)

Fermentation digests (h)	Rha	Ara	Xyl	Man	Gal	Glc	UA	Total
	Sugars in insoluble fraction							
Insolubles after ECRP addition	46	20	98	61	45	90	31	57
0.25	62	23	86	63	50	93	25	57
2	53	9	65	42	32	59	8	34
4	49	6	64	34	26	48	12	30
24	44	3	34	14	20	14	6	12
	Sugars in soluble fraction							
Solubles after ECRP addition	54	80	2	39	55	10	69	43
0.25	15	5	14	9	15	2	25	12
2	9	0	9	7	8	6	14	9
4	23	0	0	6	10	2	5	4
24	13	0	1	5	6	3	3	3
% Disappearance		44	97	65	81	74	84	92

Rhamnose (Rha), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

The utilization of ECRP insoluble fibers (45pp) (calculated from table 4.6) after 24 h was lower compared to CRP insoluble fibers (80pp) (calculated from table 4.5). This contributed to a lower overall extent of ECRP fermentation than CRP fermentation. Insoluble fibers are usually considered to be less fermentable than soluble fibers (*9*) and high levels of soluble fibers in ECRP programmed bacteria to ferment soluble fibers rather than insoluble fibers. For CRP fermentation, the bacteria were programmed to ferment insoluble fibers (during FAP) since insoluble fibers were dominant in CRP. This can also

be seen after FAP and fermentation of ECRP compared to CRP. These observations are also in agreement with lower SCFA levels for ECRP compared to CRP.

4.4. Conclusions

The increased fermentability of CRP insoluble fibers compared to ECRP insoluble fibers due to specific adaptation of the microbiota resulted in an overall increased utilization of CRP fibers than ECRP fibers.

4.5. Acknowledgements

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Chapter 5

Characteristics of bacterial enzymes present during *in vitro* fermentation of chicory root pulp by human faecal microbiota

Submitted for publication

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Abstract

Research was conducted to understand cell wall polysaccharide (CWPs) degradation in chicory root pulp (CRP) during an *in vitro* fermentation by human faecal inoculum. Enzyme activities were identified towards PNP-glycosides and polysaccharides. Enzyme extracts (EEs) from CRP fermentation liquids (2, 12 and 24 h) were incubated on polysaccharides and cell wall residues (CWRs) derived from CRP for 24 h to determine the mechanism and efficiency of enzyme action. The study showed that the presence of arabinofuranosidase, β-galactosidase, endo-arabinanase, endo-galactanase and exo-polygalacturonase increased for the 24 h EE, whereas the activity of enzymes degrading (hemi)cellulose was highest in the 12 h EE. It was hypothesized that increased levels of arabinofuranosidase, β-galactosidase, endo-arabinanase, endo-galactanase, exo-polygalacturonase, pectin de-esterifying enzymes and endo-polygalacturonase contributed to a synergy in degrading pectin in CRP from 12 to 24 h of fermentation. The higher degradability of arabinan compared to galactan in the network is due to the architecture in CRP involving more accessible arabinan than galactan.

5.1. Introduction

Dietary fibre encompasses polysaccharides and oligosaccharides that are resistant to absorption and/or digestion in the human small intestine and end up in the colon where they are fermented by the human microbiota (*1*). The microbiota can utilise fibers for fermentation by expressing a wide array of enzymes (*2*). The levels and types of enzymes expressed depend on the type of polysaccharides present during fermentation (*3*). Till date, the activity and mechanisms of the different types of enzymes involved in degrading cell wall materials containing a mixture of polysaccharides are not fully understood.

Chicory root pulp (CRP), an agricultural by-product obtained after inulin extraction, contains a mixture of cell wall polysaccharides (CWPs) (58 w/w%) especially pectin (62%), followed by cellulose (27%) and hemicellulose (11%) (*4*). The latter is mainly represented by xyloglucan in CRP (*4*). In addition to CWPs, CRP also contains remaining inulin (6 w/w%) originally present as a storage polysaccharide (*4*). Pectin in CRP is abundant in homogalacturonan (HG) (65%) followed by arabinan (19%) and galactan (11%) (*4*).

In vitro fermentation of CRP for 24 h, using human faecal inoculum resulted in the utilization of 86% arabinose, 84% galacturonic acid and 64% galactose present in the polysaccharides (*5*). The utilization rate of sugars from these polysaccharides increased after 12 h of fermentation. Although insoluble polysaccharides from the cell wall were degraded and utilized, no oligomers were found in the fermentation liquid as degradation products. Such fermentation characteristics could be due to the type and levels of bacterial enzymes expressed during fermentation of specific cell wall components (*6*). According to Jonathan et al (*6*), the level of specific enzymes may also go down during the course of fermentation.

Along with the expression of sufficient levels of enzymes during fermentation, the accessibility of polysaccharides within a cell wall network towards enzyme degradation is regarded to be crucial in determining the extent to which they are degraded. The accessibility of polysaccharides for degradative enzymes is in turn determined by the architecture of the cell wall network (*7*). Sequential removal of polysaccharides from CRP using extractants of increasing severity provided information on the various

polysaccharides involved in building up the plant cell wall architecture (*4*). Removal of a polysaccharide from a cell wall network can alter the porosity of the network and the CWP arrangement, and may increase the accessibility of other polysaccharides within the network towards enzymatic degradation (*8*).

In this study, our purpose is to characterize bacterial enzymes as present during CRP fermentation by human faecal microbiota and elucidate if CWP degradation by bacterial enzymes is affected by the accessibility of polysaccharides in the network.

5.2. Materials and Methods

Substrates

Residues obtained from sequential extraction of CRP were used to represent different cell wall structures of CRP (*4*). Residues included were Water Un-extractable Solids (WUS), Chealting Agent Un-extractable Soldis (CHUS), Dilute Alkali Un-extractable Solids (DAUS) and Concentrated Alkali Un-extractable Solids (CAUS), which all have been described previously (*4*). Commercially available polysaccharides were sugar beet pectin (CP Kelco; Lille Skensved, Denmark), linear and branched arabinan (British Sugar; Peterborough, United Kingdom), potato galactan (Megazyme, Bray, Ireland), oat spelt xylan (Sigma-Aldrich), tamarind xyloglucan (Dainippon Pharmaceutical; Osaka, Japan), locust bean gum (SKW Biosystems Benelux, Gent, Belgium), cellulose (Vitacel LC200) (J. Rettenmaier & Söhne, Rosenberg, Germany). The constituent monosaccharide composition of the substrates is presented in table 5.1.

Protein preparation from the fermentation digests

Chicory root pulp (CRP) was anaerobically fermented in a batch model for 24 h at 37°C, pH 5.8 using a standardised human faecal inoculum as described earlier (*5*). Fermentation enzymes were obtained from 1 mL of CRP fermentation digests (2, 12 and 24 h). The digests were centrifuged (20,000 x g; 10 min; 4 °C) and the supernatant containing soluble proteins was collected. The residue was suspended in buffer (25mM MES buffer pH 5.8 including 1mM PMSF, 1mM DTT, 1 mM EDTA and 50 mM NaCl) (*6*) to obtain a final volume of 1 mL as was originally present in digest before centrifugation. The cells in the suspension were kept cold by using an ice- water bath and were disrupted by a digital

sonifier (Branson, Danbury, CT, USA). The sonification was set to 30% amplitude and the treatment was performed 4 times for 30 sec with 40 sec intervals.

Table 5-1. Constituent monosaccharide composition of chicory root pulp (CRP), fractions therefore and commercial substrates

Substrates	Carbohydrate yield	Mol %								Total	DM	DA
	[g per100g in pulp]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA	% w/w	%	%
CRP	100	1	0	15	4	4	7	31	38	64	70	43
WUS	87	1	0	12	4	2	7	33	40	68	70	46
CHUS	52	1	0	10	6	3	7	53	19	59	52	91
DAUS	45	1	0	9	8	3	7	61	11	62		
CAUS	39	1	0	10	4	1	7	67	10	67		
	References											
Beet pectin	(*9*)	5	0	7	1	2	11	0.4	73	59	57	15
Linear arabinan	(*10*)	4	0	56	0	0	19	7	14	100		
Branched arabinan	(*10*)	4	0	67	0	0	14	5	10	100		
Potato galactan	Megazyme	4	0	9	0	0	78	0	9	95		
Oat spelt xylan	Sigma			15	75			10		n.a.		
Cellulose	(*11*)	0	0	0	15	1	0	81	1	99		

DM/DA: Degree of methyl/acetyl esterification expressed as moles of methanol esters /acetyl groups per 100 moles uronic acid respectively, n.a: not available. Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA).

The proteins released were recovered in the supernatant after centrifugation (20,000 x g; 10 min; 4 °C). The supernatants were combined (2 mL) and the solution was ultra-filtered using a centrifugal filter device (Amicon Ultra 10 kDa cutoff, 60 min, 4 °C; Millipore;

Cork, Ireland). The retentate was reconstituted to the original volume (2 mL) using 100 mM NaOAc buffer, pH 5.8 and used for further analysis.

Incubation of substrates with fermentation enzymes

A stock solution of each soluble substrate was prepared - 5mg/mL (100 mM NaOAc buffer, pH 5.8) and distributed into eppendorf tubes (250 μL). Insoluble substrates (oat spelt xylan, tamarind xyloglucan, cellulose and WUS, CHUS, DAUS and CAUS) were weighed directly into individual eppendorf tubes and then individual suspensions (5mg/mL) were obtained by adding the buffer as mentioned. The enzyme extract (50 μL) was added to the solution (250 μl) and incubated at 37 °C. Suspensions of polysaccharides were incubated for 30 min and 24 h and suspensions of cell wall residues were only incubated for 24 h. After incubation, the samples were heated at 100 °C for 5 min for enzyme inactivation. Substrate blanks were prepared by substituting the volume of enzyme extract with the amount of NaOAc buffer and enzyme blanks were prepared by adding the enzyme extract to the buffer without substrate. Supernatants were obtained after centrifugation (10min, 18,000 x g, 24°C) and used further for analysis.

Incubation of substrates with a cocktail of enzymes

WUS, CHUS, DAUS and CAUS were weighed in tubes and 100 mM NaOAc buffer, pH 5.5 was added to attain a concentration of 5 mg/mL. 1 μL each of endo-polygalacturonase (endoPG, EC 3.2.1.15, from *Kluyveromyces fragilis*, 0.068 U,(*12*)), pectine lyase I (PL, EC 4.2.2.10, from *Aspergillus niger*, 0.0154 U, (*13*)), endo-arabinanase (endoAra, EC 3.2.1.99, from *Chrysosporium lucknowense*, 0.006 U, (*10*)), endo-galactanase (endoGal, EC 3.2.1.89, from *Aspergillus niger*, 0.0014 U, (*14*)) and a commercial enzyme preparation Celluclast 1.5L (from *Trichoderma reesei*, 0.06 U based on the activity of CMCase in celluclast (*15*)) (Novozymes; Bagsvaerd, Denmark) were added individually to the substrate. The incubation was set at 40 °C for 24 h after which the samples were inactivated by heating at 100 °C for 5 min. Substrate blanks were prepared by only adding the substrate to NaOAc buffer (5 mg/mL) and enzyme blanks were prepared by only adding 1 μL of all the enzymes to 1 mL of NaOAc buffer. Supernatants were obtained after centrifugation (10min, 18,000 x g, 24°C) and used further for analysis.

Analytical methods

Glycosidase activity towards p-nitrophenyl (PNP) glycosides

Activities of glycosidases present in the protein extract obtained from digests were measured using PNP glycosides. PNP-α-L-galacturonide, PNP-α-L-rhamnopyranoside, PNP-α-L-arabinopyranoside, PNP-α-L-arabinofuranoside, PNP-α-D-galactopyranoside, PNP-β-D-galactopyranoside, PNP-β-D-glucopyranoside, PNP-β-D-mannopyranoside and PNP-α-D-xylopyranoside were obtained from Sigma-Aldrich (St. Louis, MN, USA). 20 μL of the protein extract was added to 100 μL of the 1mM PNP-glycosides in a microtitre plate and incubated at 37 °C. The absorbance was measured at 405 nm every 3 min for 2 h. The concentration of p-nitrophenol at every time point was determined from a standard curve (0-500 μM p-nitrophenol). This was used to calculate the enzyme activity expressed as nmol of p-nitrophenol released in 1 min by enzymes (mU) extracted from 1 mL of the fermentation digest.

PAHBAH reducing sugar assay

The reducing sugar assay using 4-hydroxybenzoic acid hydrazide (PAHBAH) was performed (*10*) on defined substrates treated with the protein extract for 30 min. Reducing sugars were quantified using a standard curve (0 - 250 μg/mL) of monosaccharides. Galacturonic acid, arabinose, galactose, mannose and xylose were used as standards for sugar beet pectin, linear and branched arabinans, potato galactan, galactomannan and xylan, respectively. Glucose was used as a standard for cellulose and xyloglucan. The enzyme activity was expressed as the amount of reducing sugars (nmol) released in 1 min (mU) by enzymes extracted from 1 mL of the fermentation digests.

High Performance Size Exclusion Chromatography

Digests were centrifuged (10min, 18,000 x g, 24°C) to obtain the soluble fraction which was analysed for molecular weight distribution using High Performance Size Exclusion Chromatography (HPSEC) on a Dionex Ultimate 3000 HPLC (Dionex, Sunnyvale, CA, USA) as described before (*4*). Three TSK-Gel columns connected in series (4000-3000-2500 SuperAW; 150 x 6 mm) were used for the analysis. They were preceded by a TSK Super AW-L guard column (35 x 4.6mm). All columns were from Tosoh Bioscience (Tokyo, Japan). Molecular mass distribution of polysaccharides was calculated using a

calibration curve of pullulan standards in the molecular mass range of 0.18 - 790 kDa (Polymer Laboratories, Palo Alto, CA, USA).

Oligosaccharide profiling

HPAEC was performed on a 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 50 mm). The soluble fraction of the substrate digests were obtained by centrifugation (10min, 18,000 x g, 24°C). Ten μl of the soluble fraction and blanks was injected into the system using a Dionex ICS5000 autosampler. The system was equipped with pulsed amperometric detection. The flow rate was 0.3ml/min. The gradient used was as described elsewhere (*11*). Xyloglucan oligomers were analysed using a gradient as described elsewhere (*16*). A standard of xyloglucan oligomers (Megazyme, Bray, Ireland) was used for identification. The software used was Chromeleon version 7 (Dionex).

Constituent monosaccharide composition

The constituent monosaccharide composition of supernatants obtained after digestion with the enzyme cocktail was determined after a methanolic HCl and TFA hydrolysis (*17*). The monosaccharides were analysed in HPAEC equipped with post column alkali addition as described elsewhere (*18*).

5.3. Results and discussion

Type and levels of enzymes in the fermentation digests

Enzymes in enzymes extracts (EEs) from 2, 12 and 24 h of CRP fermentation digests were studied for their activity on PNP-glycosides as well as defined polysaccharides as shown in table 5.2. Activities are expressed in mU per 1 mL of fermentation liquid.

Table 5-2. Enzyme activities of glycosidases and polysaccharide degrading enzymes (mU) from 1 mL fermentation digest

EE (h)		Activity towards PNP glycosides							
		α-GalA	α-L Rha	α-L Arap	α-L Araf	β-D Gal	β-D Glc	α-D Xyl	β-D Man
2	Mean	0	0	0	0	40.6	0	0	0
	SD	0	0	0	0	1.4	0	0	0
12	Mean	5.4	1.8	0	120	98.3	13.7	10.9	1.1
	SD	7.7	2.6	0	12.2	2.9	2.4	15.5	1.5
24	Mean	0	0	0	203.4	236.6	0	0	0
	SD	0	0	0	5.6	86.9	0	0	0
		Activity towards polysaccharides							
		BP	LA	BA	PG	OSX	XG	M	C
2	Mean	0.7	0.1	0	0	0.1	0	0	0
	SD	0.2	0.2	0	0	0	0	0	0
12	Mean	1.2	0.3	0.7	0.5	0.4	0	0	0
	SD	0.1	0.4	0	0.5	0.3	0.1	0	0
24	Mean	2	1.3	0.7	2.4	0.1	0.2	0	0
	SD	0.3	1.1	0.1	0.1	0	0	0	0

α-galacturonide (α-galA), α-rhamnopyranoside (α-rha), α-arabinopyranoside (α-arap), α-arabinofuranoside (α-araf), β-galactopyranoside (β-gal), β-glucoopyranoside (β-glc), α-xylopyranoside (α-xyl), β-mannopyranoside (β-man), Sugar beet pectin (BP), linear arabinan (LA), branched arabinan (BA), potato galactan (PG), Oat spelt xylan (OSX), Xyloglucan (XG), Galactomannan (M) , cellulose (C) and standard deviation (SD)

Enzyme activities towards PNP-glycosides

β-galactosidase was the only activity found in the 2 h EE (41 mU). In the 12 h EE, activities towards all PNP-glycosides were seen except for arabinopyranoside, illustrating an increase in the diversity and level of glycosidases along CRP fermentation. The highest activities were seen for arabinofuranosidase (120 mU) followed by β-galactosidase (98 mU) (table 5.2). In the 24 h EE, levels of arabinofuranosidase and β-galactosidase increased to 203 mU and 237 mU, respectively, while the levels of other glycosidases decreased. This could result from either the consumption of such glycosidase-proteins during fermentation by the microbiota as was seen during fermentation of resistant starch in the large pig intestine (*6*) or from instability of the glycosidases during fermentation.

Enzyme activities towards polysaccharides

The enzyme activities towards polysaccharides are lower compared to those towards PNP-glycosides (table 5.2). Glycosidases comprise exo-glycosidases, which cleave from the

non-reducing end of a polymer, as well as glycosidases active towards oligomers in an exo fashion. Since the PAHBAH assay only determines reducing ends, the higher levels of glycosidase activities towards PNP substrates than activities determined from the PAHBAH assay suggest higher activities of exo-glycosidases, which have specificity towards PNP-glycosides than enzymes degrading polysaccharides.

The 2 h EE showed activity towards sugar beet pectin (SBP) (0.7 mU) (table 5.2). As HPAEC revealed the presence of monomeric arabinose (Ara) and monomeric galactose (Gal) and no galacturonic acid (GalA), the measured activity by the PAHBAH assay mainly included activities of arabinofuranosidase and β-galactosidase. In 12 h EE and 24 h EE, the activity towards SBP were higher (1.2 mU) and (2 mU) and also involved exo-polygalacturonase activity revealed from the release of monomeric GalA (HPAEC analysis, data not shown).

Galactanase activity increased from 12 h EE (0.5 mU) to 24 h EE (2.4 mU)(table 5.2).

Higher activity towards branched arabinan (0.7 mU) compared to linear arabinan (0.3 mU) in the 12 h EE suggests that arabinofuranosidase is more active than endo-arabinanase in the 12 h EE. However, the higher activity towards linear arabinan (1.3 mU) compared to branched arabinan (0.7 mU) in the 24 h EE suggests that endo-arabinanase is more active in the 24 h EE than arabinofuranosidase.

In addition, the presence of xylan degrading enzymes in the 12 h EE were indicated from 0.4 mU activity measured on oat spelt xylan (table 5.2). This activity may also represent arabinofuranosidase activity since the xylan contains Ara (table 5.1). Despite the mannosidase and glucosidase activity measured towards PNP substrates, no activity was seen on cellulose and mannan in the 12 h EE (table 5.2). In the 24 h EE, barely any activities were seen for (hemi)cellulose degrading enzymes.

In summary, the 12 h EE had activities of all enzymes towards PNP glycosides, SBP, arabinan (linear and branched), galactan and xylan. The 24 h EE contained mainly increased activities of pectin degrading enzymes (arabinofuranosidase, endo-arabinanase, endo-galactanase, β-galactosidase and exo-polygalacturonase). Extended degradation of polysaccharides by enzymes would provide information on the mechanism of polysaccharide degradation as discussed in the following section.

Degradation of polysaccharides by EE

HPSEC and HPAEC of SBP digests

SBP treated with the 2 h EE was degraded as shown from the elution profile (figure 5.1A). The elution profile of SBP treated with the 12h EE showed a higher degradation than SBP treated with the 2 h EE (figure 5.1A), whereas SBP treated with the 24 EE was degraded much more than treatment with the 12 h EE. The decrease in the intensity of the high Mw peak (345 kDa) indicated that the degradation mainly involved degradation of Ara and Gal side chains of pectin. This was also demonstrated from HPAEC analysis (figure 5.2) which showed an increase in monomeric Ara, Gal and GalA released and oligomers of Ara and Gal in different extracts. This confirmed the action of arabinofuranosidase and endo-arabinanase, β-galactosidase and endo-galactanase and exo-polygalacturonase. Although no galacturonidase activity was measured in the 24 h EE in the PNP assay (2 h incubation) (table 5.2), the release of monomeric GalA from beet pectin over a prolonged incubation time (24 h) suggested that galacturonidase was present at low levels.

SBP is highly methyl esterified and acetylated (table 5.1) while the distribution of methyl esters and acetyl groups on SBP is random (*19*). The GalA release from SBP treated with the 2 h EE was calculated to be 0.4% of all GalA, while with the 12 h EE, 4% of all GalA was released and with the 24 h EE, 24% of all GalA was released. Such a high release of monomeric GalA by exo-polygalacturonase at 24 h is only possible after partial removal of methyl and acetyl esters and some backbone degradation pointing to the presence of pectin methyl esterase, pectin acetyl esterase and also endo-polygalacturonase. Earlier studies (*20, 21*), have indicated the presence of pectin methyl esterase during fermentation of highly methyl esterified citrus pectin by the human colonic microbiota.

HPSEC and HPAEC of xyloglucan digests

The HPSEC elution profile of xyloglucan treated with the 2 h EE and 24 h EE showed a shift to a lower Mw of 150 and 58 kDa respectively suggesting limited endo enzyme cleavage of the backbone. Enzymes in the 12 h EE, however, were much more active towards xyloglucan since a shift was seen to the low Mw range (5 kDa).

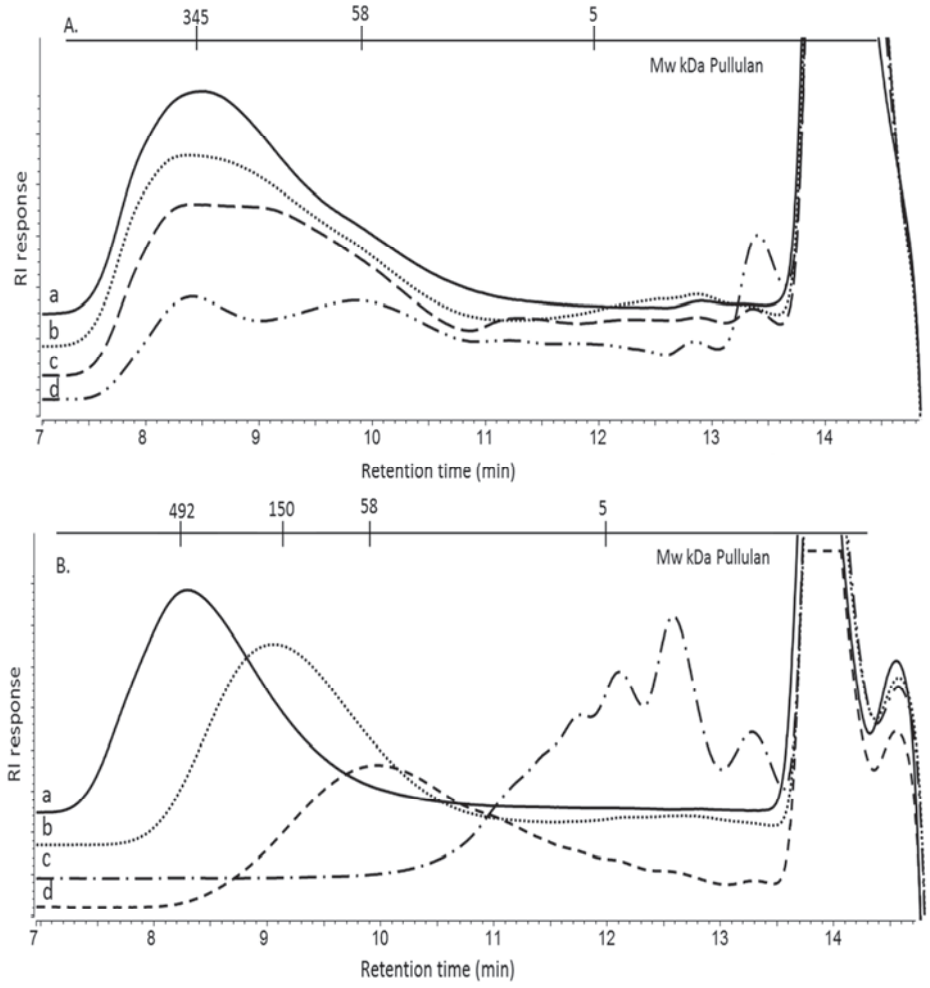

Figure 5-1. HPSEC elution patterns of **A.** Sugar beet pectin (SBP) and **B.** Xyloglucan (XG) before (a) and after incubation (24 h) with enzyme extract (EEs) obtained after 2 h (b), 12 h (c) and 24 h (d) of fermentation.

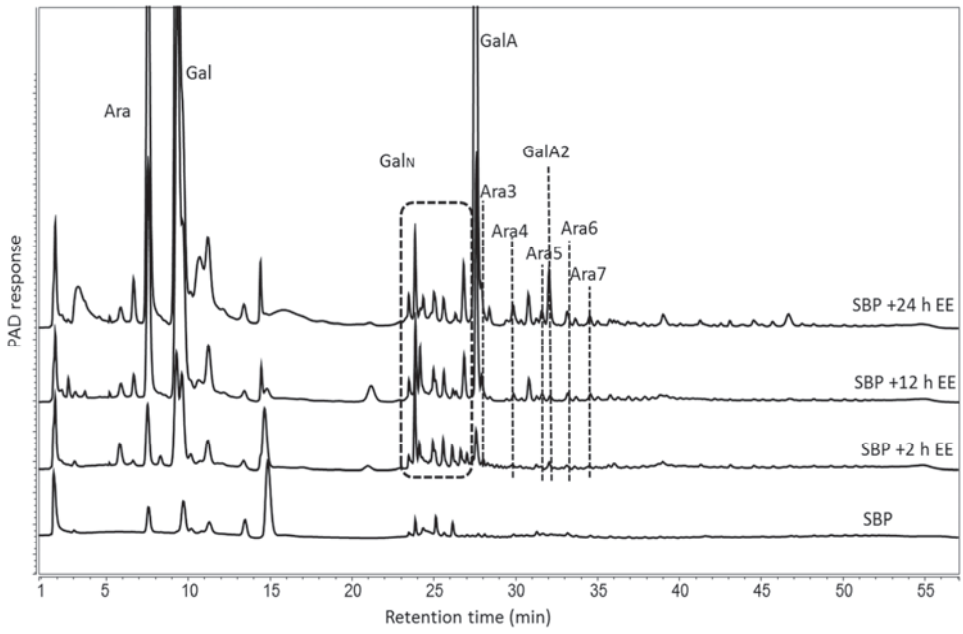

Figure 5-2. HPAEC elution profile of beet pectin (SBP) before and after incubation (24 h) with enzyme extracts (EEs) obtained after 2, 12 and 24 h of fermentation. Ara, arabinose; Gal, galactose, GalA, galacturonic acid, GalN, oligomers of Gal, Ara3, trimer of Ara

HPAEC analysis of the digests (figure 5.3) showed the release of xyloglucan oligomers treated with the 12 h EE only. The decrease of activity towards xyloglucan present in the 24 h EE is expected to be due to lack of availability of xyloglucan and utilization of xyloglucan degrading enzymes by microbiota (*6*) or due to loss in the stability of the enzymes due to no availability of the substrate. The same was observed for xylan and mannan degrading enzymes.

Compared to the colour based assays (PNP and PAHBAH), degradation studies of polysaccharides using HPSEC and HPAEC provides detailed information on the exo- or endo- acting enzymes and their specificity towards certain structures within these polysaccharides. Surprisingly, it became clear that the time point after 12 h during CRP fermentation was quite important for fiber degradation. This is because enzymes present, were able to degrade both pectin substrates (SBP, arabinan (linear and branched) and

galactan) and hemicellulose substrates (xyloglucan, xylan and mannan). After 24 h of CRP fermentation, the activity of pectin degrading enzymes increased whereas the activity of hemicellulose degrading enzymes decreased. This may explain why during CRP fermentation, sugars from pectic polysaccharides (arabinan, galactan and homogalacturonan (HG)) were utilised more than sugars from hemicellulose (*5*).

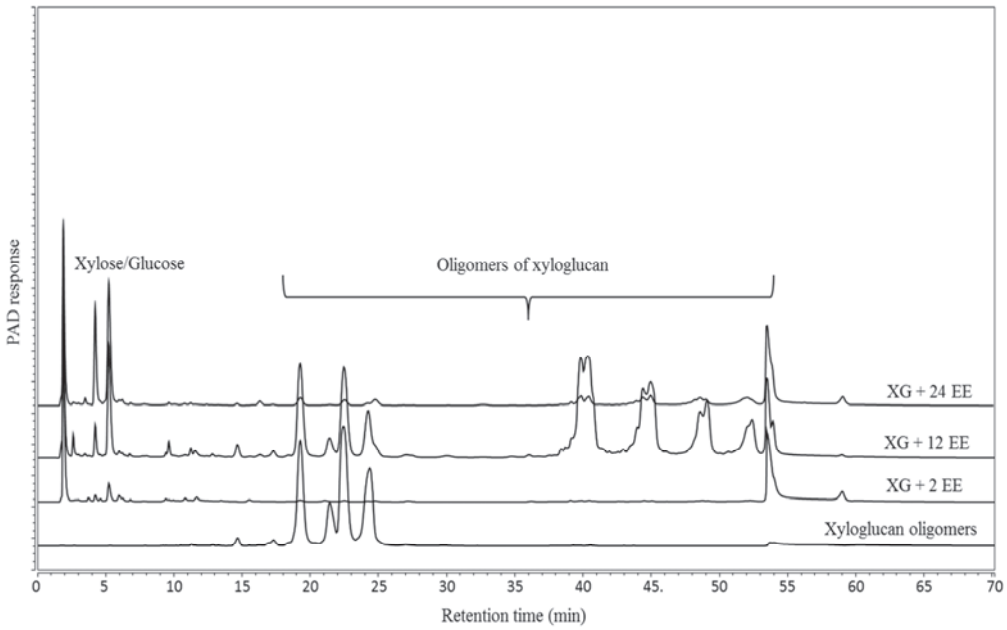

Figure 5-3. HPAEC elution profile of xyloglucan (XG) before and after incubation (24 h) with enzyme extracts (EEs) obtained after 2, 12 and 24 h of fermentation.

CRP cell wall residues

While isolated polysaccharides may be readily degraded by enzymes present, polysaccharides as present in the CRP network may only be degraded during fermentation when accessible towards enzymes. For this reason, we investigated the degradation of different cell wall residues (CWRs) derived from CRP by the three EEs from 2 h , 12 h and 24 h of CRP fermentation. These CWRs obtained by sequential removal of specific

polysaccharides may differ in the porosity of the CWP network and in the accessibility of the remaining polymer network (*8*).

CWRs were water un-extractable solids (WUS), chelating agent un-extractable solids (CHUS), dilute alkali un-extractable solids (DAUS) and concentrated alkali un-extractable solids (CAUS) which have been described previously (*4*). Differences in constituent monosaccharide composition can be seen for the different CWRs (table 5.1) and polysaccharides present in the different residues are shown in figure 5.4.

Removal of 75% HG and 50% arabinan from WUS (*4*) (figure 5.4) to yield CHUS is expected to have modified the porosity since pectin filling the space between the xyloglucan-cellulose network is known to determine the cell wall porosity (*22*). Disruption of ester bonds and further removal of half of the HG from CHUS (figure 5.4) through an alkali extraction could have opened the network further in DAUS. DAUS and CAUS are more dominant in (hemi)cellulose than CHUS (figure 5.4). Concentrated alkali removed half of hemicellulose from DAUS (figure 5.4) and the remaining insoluble network dominant in cellulose still contained hemicellulose and branched pectin (figure 5.4).

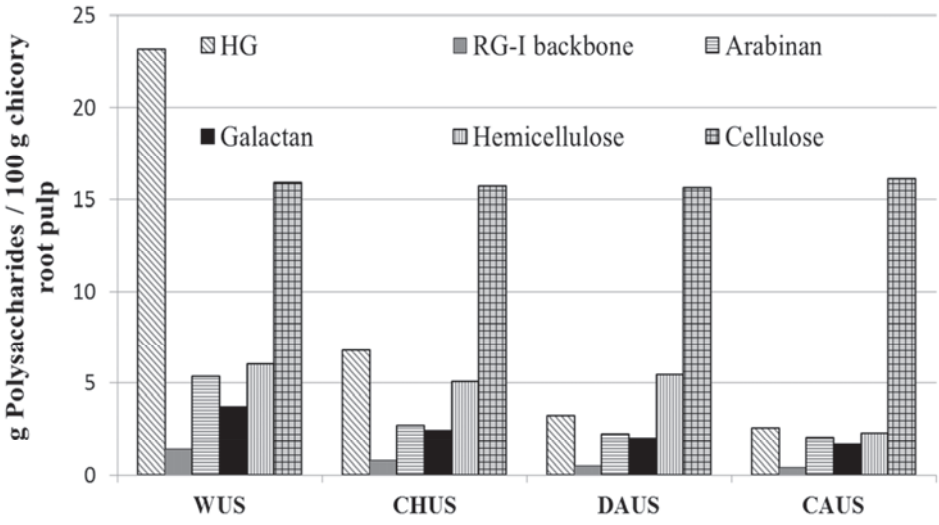

Figure 5-4. Polysaccharides (g) in residues obtained sequentially from 100 g chicory root pulp (CRP). HG, homogalacturonan (HG), RG-I (Rhamnogalacturonan). Residues are water unextractable solids (WUS), chelating agent unextractable solids (CHUS), dilute alkali unextractable solids (DAUS), concentrated alkali unextractable solids (CAUS).

Action of EE towards CRP CWRs

After 24 h incubation of the different CWRs using the EEs (2 h, 12 h and 24 h), the soluble fractions of the digests were analysed using HPSEC (data not shown). Since the elution of monosugars and oligosaccharides cannot be distinguished from proteins and salts in the low Mw range (<20 kDa), only solubilised high Mw material (polysaccharides) (20 – 600 kDa) was quantified. Only a limited solubilisation of high Mw material (polysaccharides) was seen (< 15% of substrate present). The extent and type of degradation of solubilised CWPs to oligosaccharides and monosaccharides was determined by HPAEC (figure 5.5).

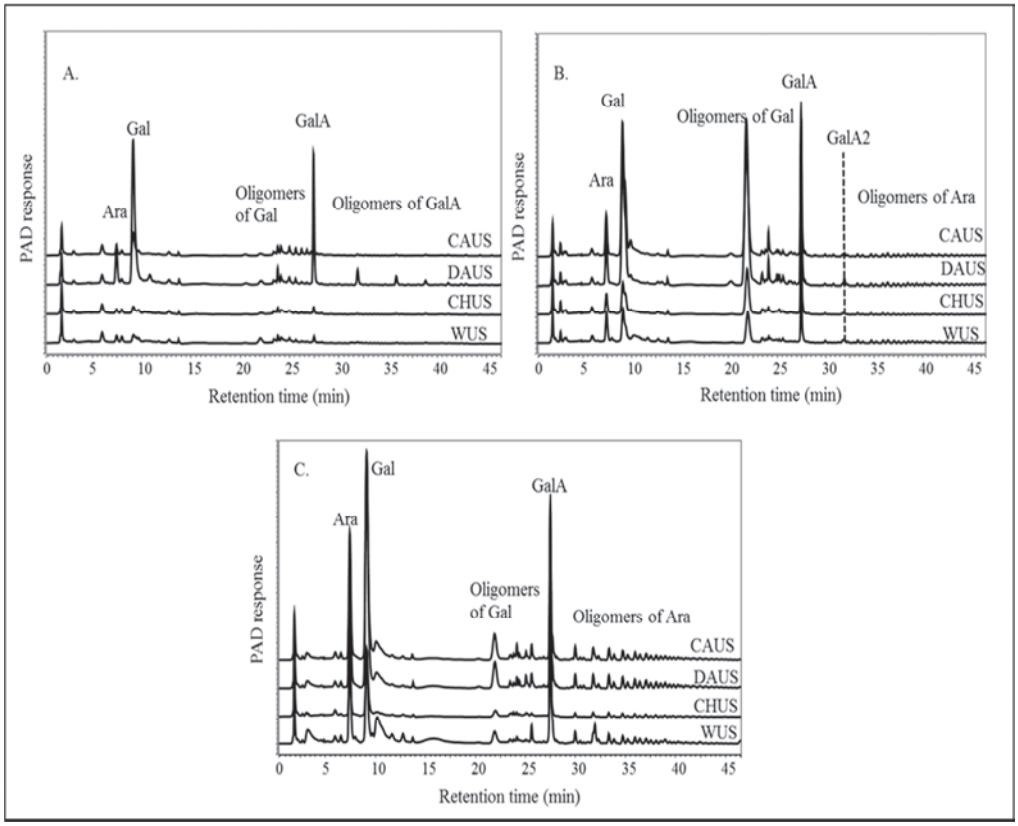

Figure 5-5. HPAEC elution profile of residues from chicory root pulp (CRP) before and after incubation (24h) with enzyme extracts (EEs) obtained after 2h (A), 12 h (B) and 24 h (C) of CRP fermentation. Residues are water unextractable solids (WUS), chelating agent unextractable solids (CHUS), dilute alkali unextractable solids (DAUS), concentrated alkali unextractable solids (CAUS). Ara, arabinose; Gal, galactose, GalA, galacturonic acid

Action of 2 h EE towards CWRs

WUS was not accessible towards 2 h EE since no release of mono/ and oligosaccharides was seen. Despite the removal of 75% HG and 50% arabinan from WUS, CHUS was also not accessible towards enzymes present in 2h EE. However, following removal of 50% HG and esters from CHUS by alkali treatment, DAUS and CAUS were both accessible towards enzymes in the 2h EE as seen from the release of mono+oligosaccharides. DAUS is more accessible than CAUS towards enzymes as shown from the higher presence of Ara, Gal and oligomers of GalA (figure 5A). This difference might be due to a different architecture of the residue in DAUS compared to CAUS. It has been suggested that xyloglucan crosslinks aid in keeping cellulose microfibrils apart (*23, 24*). It is plausible that removal of xyloglucan from DAUS caused a collapse in the hemicellulose – cellulose network making it less accessible for enzymes.

Action of 12 h EE towards CWRs

Compared to the 2 h EE, enzymes in the 12 h EE were more capable to degrade WUS and CHUS pectin populations. This is mainly due to increased activities of pectin degrading enzymes as seen above. Arabinan in WUS is more accessible towards arabinan degrading enzymes as seen from the higher presence of monosaccharides and arabinan oligosaccharides (figure 5.5B) than CHUS. This might be due to a modification in the network of CHUS after removal of HG and arabinan. Whereas the DAUS network is believed to be more open and thus accessible towards arabinan degrading enzymes than the CHUS network due to the removal of HG and esters. CAUS network, although less open than the DAUS network due to hemicellulose removal is probably relatively more open than the CHUS network and is also accessible towards arabinan degrading enzymes.

Action of 24 h EE towards CWRs

The action of 24 h EE towards CWRs resulted in an increase in the release of mono+oligosaccharides compared to the 12 h EE (figure 5.5C). The release of Ara oligomers is higher than the release of Gal oligomers (figure 5.5C), despite the higher activities of the 24 h EE towards potato galactan compared to linear arabinan (table 5.2). It can be concluded that arabinan and galactan present in the residues are differently accessible for enzymes.

The degradation of CWPs by enzymes from EEs obviously depends on the type and levels of enzymes present. However, also the way how polysaccharides are present within the cell wall architecture determines degradability to a large extent. To underline and further investigate the accessibility of the CWRs, a mixture of well-defined enzymes is used.

Accessibility of CWPs towards model enzymes

As shown in table 5.3, WUS containing high levels of calcium bound pectin and esterified pectin has limited accessibility towards the model enzymes (PG, PL, celluclast, endoAra and endoGal) as seen from only 38% GalA solubilisation. Despite this, arabinan (66%) and galactan (47%) are solubilised to a higher extent indicating that the limited accessibility of calcium bound pectin and esterified pectin does not fully affect the accessibility of arabinan and galactan.

Table 5-3. Solubilisation (%) of individual sugars from different residues obtained from chicory root pulp using an enzyme cocktail (endopolygalacturonase, pectin lyase, endoarabinanase, endogalactanase and Celluclast)

	% Solubilsation of individual sugars								
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Total
WUS	0	0	66	39	89	47	61	38	49
CHUS	0	0	63	30	53	41	48	54	49
DAUS	0	0	60	41	61	50	67	92	65
CAUS	0	0	72	59	96	56	63	97	67

Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Galacturonic acid (GalA), Water unextractable solids (WUS), chelating agent unextractable solids (CHUS), dilute alkali unextractable solids (DAUS), concentrated alkali unextractable solids (CAUS).

Removal of pectin (figure 5.4) and disruption of ester bonds from CHUS yielded DAUS and CAUS CWRs having more open space in the network. This is a plausible explanation for the increased accessibility of residual unesterified pectin as shown from the high solubilisation of almost all GalA from DAUS and CAUS (table 5.3) compared to WUS and CHUS.

Except for pectin and arabinan, all other polysaccharides were solubilised to a lesser extent from CHUS compared to the other CWRs (table 5.3). This suggested that the removal of HG and arabinan from WUS likely resulted in partial shrinking of the network and reducing the accessibility of polysaccharides (except arabinan and HG) in CHUS towards the model

enzymes. The unchanged accessibility of arabinan and HG might be due to the location of these polysaccharides in the modified network.

Arabinan was found to be more accessible for model enzymes than galactan since Ara was solubilised to a higher extent compared to Gal for all residues (table 5.3). Galactan, reported to be unbranched in CRP *(25)*, is believed to be more accumulated within the entangled network than arabinan in the cell wall since the ratio of Ara:Gal decreased from 3:1 for calcium bound pectin to 1.5:1 for pectin associated with cellulose (CAUS). This suggested that arabinan is located more to the accessible part of the cell wall compared to galactan.

Understanding fiber degradation by fermentation enzymes

As shown by the model enzymes, degradability of HG in WUS and CHUS was not affected by the degradation of arabinan and galactan. This suggests that the increased degradability of HG in WUS and CHUS by enzymes from the 12 h EE and 24 h EE is due to the increased activities of endo-polygalacturonase and pectin de-esterifying enzymes as was demonstrated towards SBP. Likewise, the increased degradation of arabinan and galactan from WUS and CHUS is due to the increased levels of arabinan and galactan degrading enzymes. This suggests that the architecture of WUS and CHUS does not affect the degradability by enzymes present in the EE.

The architecture of DAUS and CAUS does have an effect on enzyme degradability especially towards enzymes in the 2 h EE. The networks are relatively more open than WUS and CHUS due to the removal of considerable amounts of pectin and esters.

The hypothesized partial shrinkage in the network of CHUS as demonstrated by the model enzymes may be responsible for the inaccessibility of arabinan in CHUS towards arabinan degrading enzymes in the EE. Arabinan was degradable from CHUS by model enzymes because endoAra from the enzyme cocktail was more effective in degrading arabinan compared to the 2 h EE.

The larger effect of 24 h EE on arabinan than on galactan is due to the greater accessibility of arabinan than galactan in the network towards 24 h EE

5.4. Conclusions

Since WUS is similar to CRP in terms of sugar composition, the conclusions drawn from this study on enzyme activities towards WUS are used to exemplify enzyme activities towards CRP during CRP fermentation. After 2 h of CRP fermentation, enzyme levels (arabinofuranosidase and β-galactosidase) were too low to act on CRP. From 2 to 12 h, not only did the enzyme levels increase, but other enzymes (exo-polygalacturonase, endo-arabinanase, endo-galactanase) and enzymes from circumstantial evidence (pectin de-esterifying enzymes, endo- polygalacturonase) were active contributing to synergy in the degradation of HG, arabinan and galactan. From 12 to 24 h, the increased degradation caused increased porosity in the network leading to increased accessibility and degradability of residual HG, arabinan and galactan by increased levels of pectin degrading enzymes. Still, the higher degradability of arabinan compared to galactan in the network is due to the architecture in CRP involving more accessible arabinan than galactan. This contributes to the higher utilization of Ara (86%) than Gal (64%) after 24 h of CRP fermentation observed in an earlier study *(5)*.

5.5. Acknowledgements

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Chapter 6

General discussion

6.1. Motivation and aim of the research

The project titled “Novel Food Fibers” was aimed to substantiate the use of fibers from pectin rich agricultural by-products such as chicory root pulp (CRP), for food use. Main research areas involved the chemical and techno-functional characteristics of fibers in CRP, the *in vitro* and *in vivo* effects of CRP and the immune-modulatory effects of defined fibers. As part of the project, the research described in this thesis aimed at understanding the relationship between the composition of CRP cell wall polysaccharides (CWPs) including their arrangement in the cell wall network and the *in vitro* fermentability of CWPs. Soluble and insoluble fibers in CRP fermentation digests were characterised. The effect of ensiling of CRP on the solubility of fibers and the degradation of soluble and insoluble fibers during fermentation using human facecal inoculum was also studied. The levels and type of enzymes involved during the fermentation of CRP were investigated.

6.2. Cell wall polysaccharides and architecture in CRP

For the first time, characterization of CRP CWPs revealed that xyloglucan in CRP was acetylated. Also, the rhamnogalacturonan-I of pectin was mostly branched with arabinan. Besides, arabinan and galactan (30% of CRP arabinan and galactan) were suggested to be associated with cellulose.

Esterification of pectin:

CRP pectin has a high degree of methyl esterification (DM) of 70 (table 6.1) since the DM is more than 50. Pectin soluble in hot water is highly methyl esterified (DM 90) and is also highly acetylated (degree of acetylation (DA) 27), which was not known before. Chelating agent soluble pectin is also highly methyl esterified and acetylated (DM 49 and DA 14). Pectin in CHUS is highly methyl esterfied (DM 52) and acetyl substitutions (constituting 57% of all acetyl in CRP) are located on both pectin and xyloglucan. This suggests that pectin populations are present with totally different levels of ester substitutions.

Different polysaccharide populations were studied by sequentially solubilising them with extractants of increasing severity (*1*) (chapter 2). Figure 6.1 shows the distribution of different polysaccharide populations in residues obtained sequentially from CRP. Based on this information, a hypothetical distribution of polysaccharides in cell wall residues is proposed (figure 6.2).

Table 6-1. Constituent monosaccharide composition of chicory root pulp (CRP) and ensiled CRP (ECRP)

	Mol %								Total Sugars	DM	DA
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic acid	% w/w	%	%
CRP	1	0	15	4	4	7	31	38	64	70	43
ECRP	2	0	15	4	3	7	31	38	57	22	49

DM/DA: Degree of methyl/acetyl esterification expressed as moles of methyl esters /acetyl groups per 100 moles uronic acid respectively. Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Uronic acid (UA)

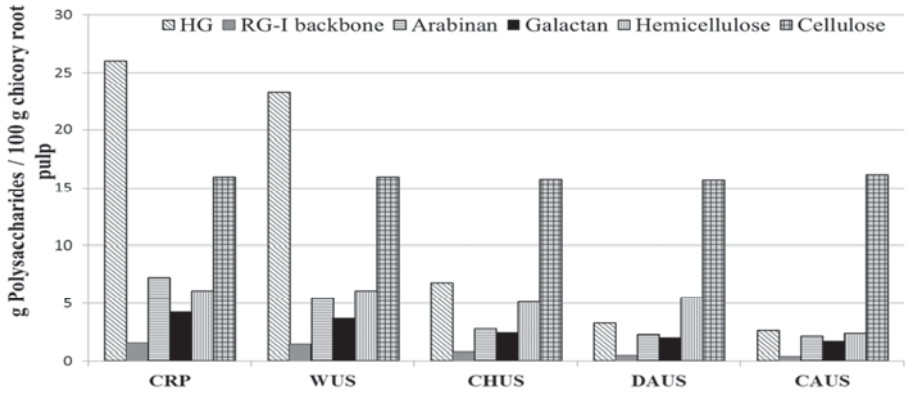

Figure 6-1. Amount of cell wall polysaccharides (g) present in chicory root pulp (CRP) and in each residue derived from sequential extractions of 100 g CRP. Water unextractable solids (WUS), chelating agent unextractable solids (CHUS), dilute alkali unextractable solids (DAUS) and concentrated alkali unextractable soldis (CAUS).

First, polysaccharides that were not strongly involved in polysaccharide entanglements were solubilised in hot water. These were inulin (6 w/w %) and arabinose rich pectin (*2*). Most of CWPs in the network were insoluble (water unextractable solids, WUS) representing 90% of CRP CWPs. Half of the insoluble pectin (55%), rich in GalA, was extractable by chelating agents (figure 6.2). Branched pectin (31% RG-I backbone, 29% arabinan and 19% galactan) was also extractable by chelatin agents suggesting that branched pectin might be part of calcium bound pectin (figure 6.2). The residue obtained after extracting pectin with chelating agents (chelating agent unextractable solids, CHUS)

was treated with dilute alkali (0.05 M NaOH) to extract pectin levels (only half of HG and 20% RG from CHUS), which were ester bound in the cell wall (figure 6.1).

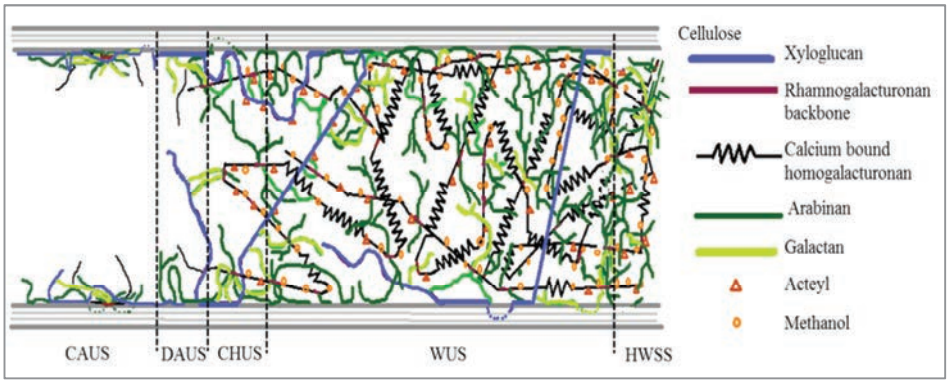

Figure 6-2. Polysaccharide distribution in the cell walls of residues derived from chicory root pulp. HWSS (Hot water soluble solids), water unextractable solids (WUS), chelating agent unextractable solids (CHUS), dilute alkali unextractable solids (DAUS) and concentrated alkali unextractable soldis (CAUS).

Compared to sugar beet pulp, CRP contains no ferulic acid as was determined for our samples by using the procedure described elsewhere (*3*).

The dilute alkali unextractable solids, DAUS, were treated with 4 M NaOH. It extracted hemicellulose dominant in xyloglucan of the XXXG type (*2*) where X represents xylose attached to glucose (G). Different types of substituted xyloglucan were identified (chapter 2). These were XLXG or XXLG in which L corresponds to XXXG substituted with galactose (Gal), XXFG and XLFG in which F corresponds to XXLG or XLLG further substituted with fucose. Upon digestion of CHUS with an enzyme cocktail (chapter 5), an additional oligomer was identified by MALDI-TOF MS (figure 6.3). This was seen from a peak corresponding to mass to charge ratio of sodium adduct of XJFG/XFJG (m/z =1717) in which J corresponds to XLFG substituted with Gal. This new repeating xyloglucan unit may exist as such or the XXFG fragment may originate from a covalently lined RG-I-galactan segment to xyloglucan as reported elsewhere *(4, 5)*.

As was not known before, we found xyloglucan to be acetylated as observed from MALDI TOF-MS analysis of xyloglucan oligomers (figure 6.3) obtained from digestion of CHUS with an enzyme preparation containing endoglucanase (chapter 5). This was seen from a peak corresponding to a sodium adduct of acetylated XXFG (m/z = 1435) and acetylated

XLFG (m/z = 1597) (figure 6.3). The acetyl group may be present on Gal or on an unbranched glucose residue (*6*).

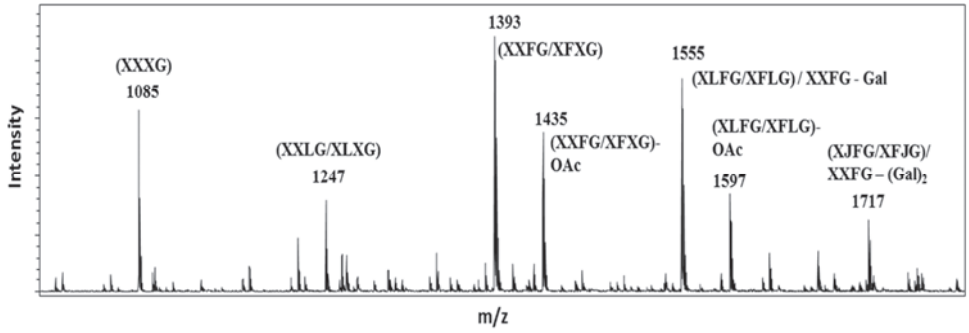

Figure 6-3. MALDI-TOF mass spectrum of xyloglucan oligomers obtained after treatment of CHUS (chelating agent un-extractable solids) with an enzyme cocktail (polygalacturonase, pectin lyase, endo-arabinanase, endo-galactanase and celluclast). X, xylose, G, glucose, L, galactose (Gal) attached to X in XXXG; F, fucose attached to galactose in XXXG; J, galactose attached to galactose in XXXG.

Differences were seen in xyloglucan populations released from the residues upon digestion with an enzyme cocktail (polygalacturonase, pectin lyase, endo-arabinanase, endo-galactanase and celluclast). For example, the ratio of XJFG: XXXG (figure 6.3) was higher for CHUS (0.3:1) and DAUS (0.5:1) compared to CAUS (0.07:1). This indicated slight differences present in the various xyloglucans extracted.

Quite some pectin (17%) was still present in the concentrated alkali unextractable solids, CAUS (figure 6.1) (Chapter 2). The pectin side chains can be hypothesized to be associated with cellulose (figure 6.2) as was observed for sugar beet pulp (*7*). These may be either absorbed on cellulose fibers or physically entrapped within the hemicellulose and cellulose network. Pectic polysaccharides associated with cellulose can be released using cellulases degrading the cellulose network *(8)*. However, digestion of CAUS containing alkali swollen cellulose with an enzyme cocktail containing a mix of glucanases and cellulases, as described in chapter 5, did not remove all pectic sugars from the network. The residual network still contained some RG as indicated from arabinose (8 mol%), galactose (9 mol%), GalA (1 mol%) and rhamnose (3 mol%) and hemicellulose (xylose, 5 mol% and fucose, 1 mol%)(table 6.2). This suggested that the branched pectin and hemicellulose in the network were not degraded due to their inaccessibility caused by their possible entrapment between cellulose microfibrils (figure 6.2).

Table 6-2. Constituent monosaccharide composition and proportions of constituent monosaccharides present in the insoluble residue obtained after treating conc. alkali un-extractable solids (CAUS) from chicory root pulp (CRP) with an enzyme cocktail (Chapter 5)

	Mol % Insoluble polysaccharides								Insolubles
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	from 5000 ug CAUS
Insoluble residue	3	1	8	5	0	9	74	1	1103
	% CAUS monosaccharides in the insoluble fraction								
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Total*
Insoluble residue	100	100	28	41	4	44	37	3	33

*Proportion of CAUS carbohydrates in the insoluble fraction. Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Galacturonic acid (GalA)

6.3. Disruption of chicory root pulp cell wall

The water holding capacity (WHC) of CRP was 6 mL/g, and found to arise from the network consisting of alkali soluble pectin, xyloglucan and mannan representing 9%, 2.5% and 0.9% of CRP carbohydrates, respectively (Chapter 2).

CRP was ensiled in order to lower the WHC of the pulp (Chapter 2). Reduction in the WHC was necessary to aid supplementation of CRP to dry food substrates. Due to ensiling, the high WHC of CRP was reduced by 44%. The ensiled CRP (ECRP) had a similar constituent monosaccharide composition and CWP content as CRP (chapter 2). As a consequence of ensiling, ECRP had four times more pectin in the soluble fraction compared to CRP and ECRP pectin had a DM of 22 compared to 70 for CRP pectin. Furthermore, the insoluble cell wall network in ECRP was hypothesized to be more open than CRP due to the degradation of calcium bound pectin + alkali soluble branched pectin and the increased solubilisation of hemicellulose in alkali compared to CRP (Chapter 2) (figure 6.4). In addition, branched pectin associated with cellulose in CRP (17% of CRP) (*2*) was also solubilised from ECRP since less branched pectin remained in CAUS ECRP (7%)(*2*). The lower WHC for ECRP CAUS compared to CRP CAUS (chapter 2) suggested that pectin bound to cellulose was also involved in the WHC of CRP.

Thus, the entire cell wall network was disrupted which reduced the WHC of CRP.

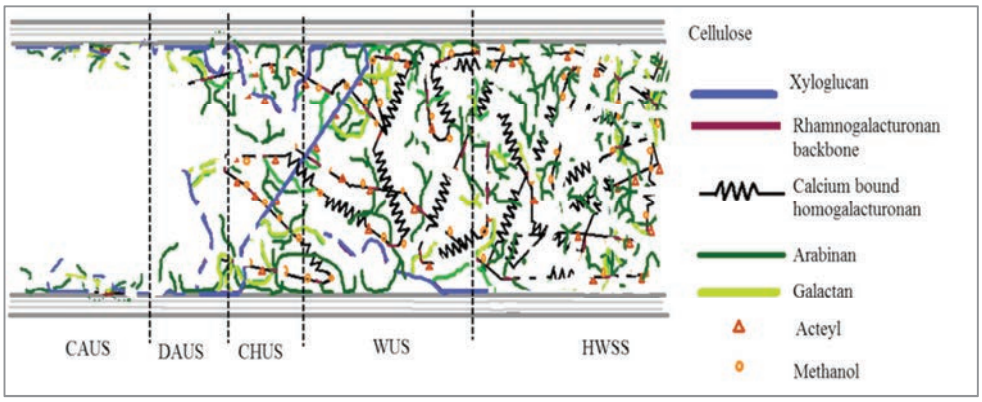

Figure 6-4. Polysaccharide distribution in the cell walls of residues derived from ensiled chicory root pulp. HWSS (Hot water soluble solids), Water unextractable solids (WUS), Chelating agent unextractable solids (CHUS), Dilute alkali unextractable solids (DAUS) and Concentrated alkali unextractable soldis (CAUS).

6.4. Cell wall polysaccharide fermentation

CRP and ECRP were well fermentable by human fecal microbiota during fermentation in both *in vitro* models viz. a closed system 'batch' model (*9*)(chapter 3) and a TNO *In vitro* Model 2 (TIM-2) (chapter 4). The latter mimics conditions of the lumen in the human proximal colon (*10*).

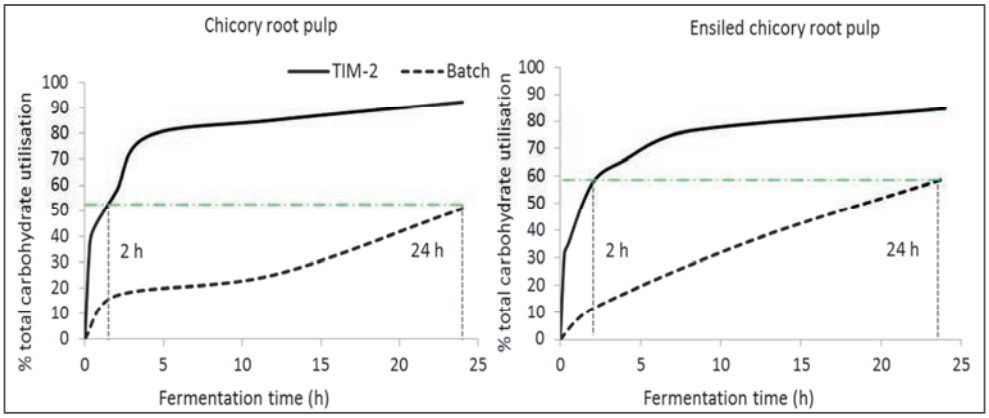

Figure 6-5. Total carbohydrate utilisation (%) in batch and TIM-2 fermentation of chicory root pulp and ensiled chicory root pulp

Figure 6.5 shows utilisation of carbohydrates in both models for both fibers. The batch fermentations are slower compared to the TIM-2 fermentations. This is because bacteria in the batch model need time to adapt to the fiber (lag phase), whereas microbiota in the TIM-2 were already adapted to the fiber for 48 h before being fermented. The microbiota concentration in TIM-2 was atleast 10 times higher than in the batch set-up and the substrate concentration was two times more than in the batch set-up (chapter 4).

Table 6-2. Constituent monosaccharide composition and sugar utilization levels of CRP and ECRP fermentation digests from batch and TIM-2

	Fermentation time (h)	Mol%							
		Rha	Ara	Xyl	Man	Gal	Glc	UA	w/w % total
CRP fermentation									
Batch	0 (blank)	1	15	4	4	9	35	32	8
TIM-2	After CRP addition (0)	3	12	7	4	8	34	32	26
Batch	24	2	4	8	4	6	65	10	4
TIM-2	2	4	5	13	4	7	47	20	15
% Batch disappearance	24	42	86	11	50	63	7	84	51
% TIM-2 disappearance	2	38	82	22	61	60	41	74	58
ECRP fermentation									
Batch	0 (blank)	2	14	4	3	8	35	34	7
TIM-2	After ECRP addition (0)	3	11	8	3	8	34	33	23
Batch	24	2	2	9	5	5	68	10	4
TIM-2	2	4	2	14	4	7	52	17	14
% Batch disappearance	24	39	95	9	36	76	22	88	59
% TIM-2 disappearance	2	38	91	26	51	60	35	78	57

Rhamnose (Rha), Fucose (Fuc), Arabinose (Ara), Xylose (Xyl), Mannose (Man), Galactose (Gal), Glucose (Glc), Galacturonic acid (GalA)

Although the extent of carbohydrate utilisation after 24 h in batch is similar to 2 h in the TIM-2 (green dotted line in figure 6.5), the constituent monosaccharide compositions between 24 h batch digests and 2 h TIM-2 digests are different for both CRP and ECRP fermentations (table 6.2) implying different polysaccharide degradation behaviour in the two models. After 24 h of CRP fermentation, mostly pectin (80%) and to a lesser extent, (hemi)cellulose (11%) was fermented in the batch model (Chapter 3), whereas in TIM-2

after 2 h, (hemi)cellulose was more extensively fermented (40%) next to pectin (72%) (Chapter 3). This indicates that fermentation in the batch model was selective towards pectin, whereas fermentation in TIM-2 involved bacteria that were also able to ferment (hemi)cellulose. Likewise, the same differences was observed for ECRP fermentation in the batch and the TIM-2.

Microbiota in CRP fermentation

The microbiota composition of the 24 h fermentation digests obtained during CRP fermentation in the batch model (chapter 3) and TIM-2 (unpublished data) is expressed as % abundance of genera of the total microbiota in the fermentation digest (figure 6.6). The 24 h digest from TIM-2 is compared with the 24 h digest of the batch fermentation since the microbiota composition for the 2 h digest in TIM-2 was not determined. Still, the microbiota composition for the 24 h TIM-2 digest is expected to be representative for the 2 h TIM-2 digest since a constant ratio of acetate: propionate: butyrate (58:23:18) was seen in the adaptation phase and the fermentation phase. Shifts in activity in TIM-2 are reported to occur swiftly and are maintained throughout the experimental period *(11).* Analysis of the microbiota composition in both model fermentations showed that both the 24 h digests contain *Bacteroides* and unclassified bacteria from the Lachnospiraceae family. However, the ratio of Bacteroides : bacteria from the Lachnospiraceae family is much higher in the batch (12:1) than in TIM 2 (1:1) fermentation (figure 6.6). This difference in proportions of dominating bacteria may contribute to differences in polysaccharide utilisation between the batch and TIM-2 fermentations. This difference also arises from adaptation in TIM-2. *Bacteroides* is a diverse group comprising many different species, which are selective towards degrading different CWPs (*12, 13*). *B. thetaiotaomicron*, a dominant member of *Bacteroides*, has been suggested to utilize pectin (*13*), whereas *Lachnospiraceae* which also adhere to fibers, have been shown to utilize hemicellulose and cellulose (*14*, *15*). This means that the lower proportion of *Lachnospiraceae* in the batch fermentation compared to the TIM-2 fermentation may be responsible for the poor utilisation of hemicellulose and cellulose. The lower presence of *Lachnospiraceae* could be due to conditions caused by metabolite accumulation during the batch fermentation.

Bacteroides were also found to be the dominant bacteria (60% abundance) in other batch fermentations involving pectin, such as fermentation of sugar beet pulp oligosaccharides using human facecal inoculum (*16*).

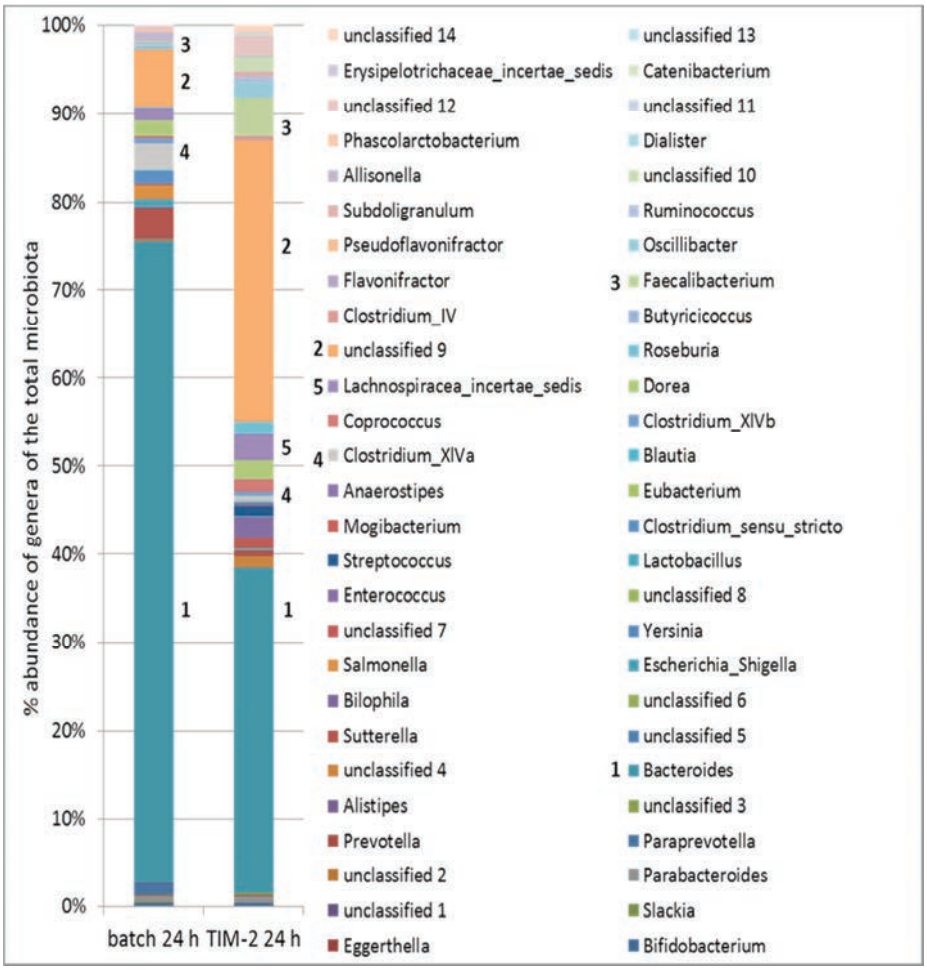

Figure 6-6. Microbiota composition during fermentation of chicory root pulp (CRP) in the batch and TIM-2, expressed as % abundance of genera of the total microbiota in the fermentation digest. Numbers in the legend indicate operation taxonomic units (OTUs) which are highlighted in the graph.

Presence of soluble fibers affect insoluble fiber fermentation efficiency

Both batch and TIM-2 fermentations showed a lower insoluble fiber utilization from ECRP compared to CRP (chapters 3 and 4). We hypothesized that in ECRP, containing equal proportions of soluble and insoluble fibers, the bacteria were programmed to utilize soluble fibers since soluble fibers are more fermentable than insoluble fibers (*9*) and the bacteria were not adapted to grow on insoluble fibers. It has been reported that different bacteria

grow on soluble fibers and insoluble fibers (*17*). This is because bacteria degrading insoluble fiber need to adhere to the fiber (*18*) whereas bacteria degrading soluble fiber do not. In a fermentation that contains more insoluble fiber than soluble fiber as in CRP, there are more bacteria able to attach and colonize insoluble fibers compared to fermentation where soluble fiber and insoluble fiber are present in equal proportions. The ECRP fermentation is hypothesized to stimulate more soluble fiber degrading bacteria than CRP. The growth of soluble fiber degrading bacteria was further enhanced during adaptation of microbiota in TIM-2 when ECRP was fed continuously in the model as is the case for insoluble fiber in CRP.

Fermentation of CRP and ECRP in both fermentations resulted in almost complete utilisation of arabinan and homogalacturonan (HG) (chapters 3 and 4) (table 6.1). As mentioned earlier, most of arabinan and HG was extractable by chelating agent. This implied that these polysaccharides were relatively easily accessible for bacterial enzymes. Although bacteria may not be able to enter the cell wall network and degrade fibers, it is advantageous for bacteria to have polysaccharide degrading enzymes in close association with the bacterial cell so that polysaccharide degradation products can be transported immediately across the cell wall (*12*). Although the mechanism of feeding is not clear, extracellular enzymes produced by bacteria may be endo-acting enzymes, which cleave polysaccharides to smaller fragments which are then available for the cell wall associated exo-enzymes to be degraded further to monomers and transported across the cell wall.

Enzyme activity in CWP degradation from chicory root pulp

Analysis of enzyme activities from fermentation liquids of CRP batch fermentation (2 h, 12 h and 24 h)(chapter 5) revealed differences in the diversity and levels of enzymes. Enzyme activities were mostly exo compared to endo. The enzyme extract (EE) from 24 h CRP fermentation had increased activities of enzymes towards pectin substrates (sugar beet pectin, arabinan and galactan) and hemicellulose substrates (xyloglucan, xylan and manna) whereas the EE from 24 h fermentation had increased activities of only pectin degrading enzymes. The decrease in the activity of (hemi)cellulose degrading enzymes may be due to their instability or utilization by bacteria arising from inavailability of (hemi)cellulose (*19*). The no availability of (hemi)cellulose may be due to the inaccessibility of (hemi)cellulose in the architecture for enzymes after 12 h of fermentation. This explains why pectin was selectively utilized during CRP batch fermentation (chapter 3).

The time after 12 h of CRP fermentation was important because of increased levels of arabinofuranosidase and β-galactosidase as well as the increased levels of other enzymes (exo-polygalacturonase, endo-arabinanase, endo-galactanase) and enzymes from circumstantial evidence (pectin de-esterifying enzymes, endo-polygalacturonase). These enzymes contributed to synergy in increasing the degradation of HG, arabinan and galactan. From 12 to 24 h, the increased degradation presumably caused increased porosity in the network leading to increased accessibility for enzymes. This combined with the increased levels of pectin degrading enzymes, contributed to an overall utilisation of GalA, Ara and Gal during CRP fermentation.

Still, the architecture of CRP seemed to cause a difference between arabinan and galactan degradation despite higher activities of the 24 h EE on potato galactan compared to linear arabinan. Chapter 5 showed that arabinan was more accessible for enzymes than galactan in the network. This contributed to the higher utilisation of Ara (86%) than Gal (64%) after 24 h of CRP fermentation observed in an earlier study *(9)*.

Enzyme activities during ECRP fermentation were not measured. However, enzymes expressed during CRP fermentation may be the same during ECRP fermentation since both CRP and ECRP have similar constituent monosaccharide compositions. Due to the presence of soluble polysaccharides in ECRP, the enzyme expression is expected to happen earlier than in CRP fermentation since the rate of polysaccharide utilisation was faster in ECRP than CRP (chapter 3).

6.5. Future perspectives

The aim of this thesis was to understand the relationship between CRP CWPs and CRP *in vitro* fermentability. Results of this thesis proved the hypothesis that a) pectin in CRP is fermentable b) increased pectin fermentability after 12 h of CRP fermentation is due to increased activities of pectin degrading enzymes. However, results of the TIM-2 study also disproved the hypothesis that a cell wall material containing more soluble fibers is more fermentable than a cell wall material containing less soluble fibers. Results of this thesis will be useful in understanding *in vitro* fermentations of cell wall materials.

Quantification of sugar disappearance was used to determine the utilisation of sugars during fermentation. In addition, determination of the degree of esterification during fermentation will be useful in order to determine how esterfied pectin is utilized during fermentation. It was hypothesized in chapter 5 that pectin methyl esterase and pectin acetyl esterase were

active during CRP fermentation in the batch model. It is also recommended to analyse activities of these enzymes for fermentations involving esterfied pectin.

Combining fiber utilisation with analysis of the microbiota composition aided in predicting which bacteria are functional on fibers. As found elsewhere (*16*) and in chapter 2, batch fermentation of pectin rich fibers promoted growth of *Bacteroides*. The technique used to determine microbiota composition in this thesis allowed analysis up to the genus level. Use of other techniques such as, phylogenetic microarray analysis (*20*) would be useful to determining the microbiota composition till the species level. This is especially necessary for fermentations which promote the growth of *Bacteroides* since this group comprises a broad distribution of species.

From chapters 2 and 5, a correlation was found between the WHC of CRP and the accessibility of residues derived sequentially from CRP. WUS and CHUS, having similar WHC, were both inaccessible towards fermentation enzymes in the 2h EE prepared from CRP fermentation. DAUS, having the highest WHC, was the most accessible for enzymes followed by CAUS. This indicates that the WHC (measure of a cell wall network to accommodate water) may be a parameter to be used to determine accessability of CWPs in residues.

The use of two different models for fermenting CRP and ECRP pointed to clear differences in fermentation behaviour between the models. Since the batch fermentation is slow, changes in polysaccharide structures could be studied during batch fermentation. The TIM-2 can be used for studying the effect of microbiota adaptation on fiber utilization. Unlike TIM-2, batch fermentation of CRP did not involve adaptation of microbiota to the fiber substrate. Still, to be compared with TIM-2, an adaptation cannot be advocated for batch fermentation, due to metabolite accumulation and changes in the microbiota composition which may not be representative as the starting bacteria in the fermentation phase.

Fermentation of CRP in TIM-2 to predict *in vivo* fermentation needs to be verified. The pH in TIM-2 is maintained at a constant pH (5.8) of the human proximal colon by alkali addition. In the human colon, the pH varies from 5.7 in the caecum to 6.7 in the rectum (*21*). Gastrointestinal movement and gut transit time *in vivo* may determine the site of fermentation of the fiber in the colon, especially if the fiber has a high WHC. CRP with a high WHC is expected to shorten the gut transit time. Consumption of 33g/day of beet fiber decreased transit time by 25% in healthy human subjects *(22)*. The rate of CRP fermentation, as seen in the TIM-2, may not be the case in *in vivo.* The SCFA ratio in TIM-

2 fermentation may also be different *in vivo* since SCFA production is also affected by the gut pH and gut transit time (*23*). However, acetate will still be the dominant SCFA since fermentation of pectin is known to produce high levels of acetate (*23*). Based on these conclusions, it is recommended to compare TIM-2 fermentation of CRP to an *in vivo* fermentation of CRP. Furthermore, the effect of adaptation in TIM-2 and adaptability *in vivo* should be compared to test if differences arise in fermentations in TIM-2 and *in vivo* because of adaptation.

From this thesis, detailed information on different polysaccharide structures as present in CRP and their likely arrangement in the cell wall was revealed. The TIM-2 fermentation revealed that cell wall materials containing more soluble fibers need not be as fermentable as cell wall materials containing insoluble fibers. Although TIM-2 mimics the proximal colon, it would be beneficial to have the whole colon in the model so as to mimic fiber dependant colon transit time.

6.6. References

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Summary

Cell wall polysaccharides (CWPs) from agricultural by-products have the potential to be used as fiber supplements to food. Chicory root pulp (CRP) is an agricultural by-product obtained after the industrial extraction of inulin from the chicory root and is aimed to be used as a dietary fiber. Not much is known on the different CWP populations, interactions between CWPs in CRP as well as the fermentability of CRP. Furthermore, solubilisation of fibers from the cell wall by processing of the pulp may have an effect on the fermentability of the pulp. The aim of this thesis was to understand the effect of CRP CWPs present as soluble and insoluble fibers on their *in vitro* fermentability by human colonic bacteria.

Chapter 1 describes CWP structures and enzymes that can degrade different polysaccharide structures. The fermentation of polysaccharides and the bacteria involved in fermenting fibers are described. In addition, the chapter also describes different fermentation models such as the batch and TNO *In vitro* Model (TIM-2) that could be used to study fermentation since the fermentation of a fiber may also depend on the type of model used.

First, CWPs of CRP and processed (ensiled) CRP (ECRP) were characterised in **Chapter 2.** CWPs of CRP comprised 62 % pectin, 11% hemicellulose and 27% cellulose. Pectin in CRP is highly methyl esterfied whereas both pectin and xyloglucan were found to be acetylated. Xyloglucans were mainly of the XXXG type. Half of CRP pectin is extractable with chelating agents. CRP CWPs are almost (90%) insoluble, whereas ECRP having similar polysaccharide content as CRP has equal proportions of soluble and insoluble CWPs. Moreover, the insoluble cell wall network of ECRP was hypothesized to be more open due to degradation of residual pectin and modification of the (hemi)cellulose network.

Chapter 3 describes *in vitro* fermentability of CRP and ECRP in a batch model using human faecal inoculum. Both CRP and ECRP were fermentable in 24 h. 51% of all carbohydrates were utilised from CRP and 59% of all carbohydrates were utilised from ECRP. *Bacteroides* were found to be the dominant bacteria in fermenting CRP and are expected to directly utilise mono and oligomeric degradation products formed from the insoluble fiber. This was concluded from the absence of degradation products and decrease in the levels of CWPs. Ensiling of CRP had more effect on the rate of fiber utilization (11% more carbohydrate utilization in 12 h compared to CRP) than on the extent of fiber

utilization. It was concluded that this effect was due to the presence of soluble fibers and the hypothesized open cell wall network of ECRP.

Fermentation of CRP and ECRP in a dynamic model such as the TIM-2 was described in **Chapter 4**. After the adaptation phase of the human faecal inoculum to CRP and ECRP for 48 h, 57% of carbohydrates were rapidly utilised in 2 h. The fermentability of CRP and ECRP were rapid compared to the batch fermentation. ECRP carbohydrates (85%) were slightly less fermented in 24 h compared to CRP carbohydrates (92%). This was due to lower utilization of insoluble fibers in ECRP compared to CRP. It was hypothesized in this study that soluble fibers that are readily fermentable and dominantly present in ECRP favoured proliferation of soluble fiber degrading bacteria more than the insoluble fiber degrading bacteria and the inflexibility of the soluble fiber degrading bacteria to adapt to the insoluble fiber thereafter. The lower SCFA levels for ECRP were in line with the lower carbohydrate utilization in ECRP than CRP.

In order to understand pectin degradation in CRP during batch *in vitro* fermentation by human faecal inoculum, enzyme activities were studied over fermentation time as described in **Chapter 5**. Enzymes activities were identified towards PNP-glycosides and complex polysaccharides. Enzyme extracts (EEs) from CRP fermentation liquids (2, 12 and 24 h) were incubated also on polysaccharides and cell wall residues (CWRs) derived from CRP for 24 h to determine the mechanism and efficiency of enzyme action. The study showed an increase in levels of arabinofuranosidase, β-galactosidase, endo-arabinanase, endo-galactanase and exo-polygalacturonase and enzymes suggested from the release of galacturonic acid (pectin de-esterifying enzymes and endo-polygalacturonase). Levels of these enzymes contributed to a synergy in degrading pectin in CRP from 12 to 24 h of fermentation. It was hypothesized that the increased degradation with time caused increased porosity in the network leading to an increased accessibility for the enzymes. This combined with the increased levels of pectin degrading enzymes, enhanced degradability of residual homogalacturonan, arabinan and galactan. The higher degradability of arabinan compared to galactan in the network was due to the architecture in CRP involving more accessible arabinan than galactan.

In **Chapter 6**, the distribution of CWPs in the cell wall is proposed for CRP and ECRP. Batch fermentation of CRP and ECRP were compared to TIM-2 fermentation of CRP and

ECRP. The influence of soluble fibers on the fermentability of insoluble fibers is discussed. The enzyme activities involved in fermenting CRP in the batch model are also discussed.

Samenvatting

Celwandpolysachariden (CWPs) uit agrarische bijproducten kunnen mogelijk gebruikt worden als vezel supplementen in levensmiddelen. Cichorei wortelpulp (CRP) is een agrarisch bijproduct dat verkregen wordt na industriële extractie van inuline uit de cichorei-wortel en wordt gebruikt als voedingsvezel. Er is nog niet veel bekend over de verschillende populaties van CRP polysachariden, hun interacties binnen de celwand en hun fermenteerbaarheid. Het in oplossing brengen van vezels uit de celwand tijdens de verwerking van de pulp kan mogelijk ook effect hebben op de fermenteerbaarheid van de vezels. Het doel van het in dit proefschrift beschreven onderzoek was dan ook het bestuderen van het effect van oplosbare en onoplosbare CRP CWPs op de *in vitro* fermenteerbaarheid door menselijke darmbacteriën.

Hoofdstuk 1 beschrijft CWP structuren en enzymen die verschillende polysacharide structuren kunnen afbreken. De fermentatie van polysachariden en de bacteriën die betrokken zijn bij de fermentatie van vezels worden besproken. Daarnaast worden verschillende fermentatiemodellen zoals het batchmodel en het TNO *In vitro* Model (TIM-2) behandeld. Hoewel beide modellen vaak gebruikt worden om fermentatie te bestuderen, is de waargenomen fermentatie mogelijk afhankelijk van het gebruikte model.

De karakterisering van de CWPs van CRP en ingekuild (ensiled) CRP (ECRP) wordt beschreven in **Hoofdstuk 2**. CWPs uit CRP bestaan voor 62% uit pectine, voor 11% uit hemicellulose en voor 27% uit cellulose. Pectine in CRP is hoog methylveresterd en zowel pectine als xyloglucaan zijn geacetyleerd. Xyloglucanen zijn vooral van het XXXG-type. De helft van de CRP pectine kan worden geëxtraheerd in de aanwezigheid van een sekwestreermiddel. De andere CRP CWPs (90%), zijn vrijwel onoplosbaar. ECRP echter bevat gelijke hoeveelheden oplosbare en onoplosbare CWPs. Het onoplosbare celwandnetwerk van ECRP wordt beschouwd als ‘meer open’ door de modificatie van het pectine en het hemicellulose-cellulose netwerk.

Hoofdstuk 3 beschrijft de *in vitro* fermenteerbaarheid van CRP en ECRP in een batchmodel, gebruik makend van humaan fecaal inoculum. Zowel CRP als ECRP werden binnen 24 uur gefermenteerd. Van CRP werd 51% van alle koolhydraten en van ECRP werd 59% van alle koolhydraten benut. Bacteroides bleken de meest dominant aanwezige bacteriën bij de fermentatie van CRP en worden geacht mono- en oligomere afbraakproducten vrij te maken uit oplosbare vezels en die meteen zelf te gebruiken. Dit

werd geconcludeerd uit de afwezigheid van afbraakproducten en afname in de hoeveelheden CWPs. Het inkuilen van CRP had vooral effect op de snelheid waarmee vezels benut werden (11% meer koolhydraten benut binnen 12 uur in vergelijking met CRP) en minder op de mate van vezel benutting. Dit effect werd mogelijk veroorzaakt door de aanwezigheid van oplosbare vezels en het geopende celwandnetwerk van ECRP.

Fermentatie van CRP en ECRP in een dynamisch fermentatiemodel, zoals TIM-2 wordt beschreven in **Hoofdstuk 4.** Na een aanpassingsfase van de fecale microbiota aan CRP en ECRP gedurende 48 uur, werd 57% van de koolhydraten binnen 2 uur benut. De fermentatie van CRP en ECRP in het TIM model was snel vergeleken met de batchfermentatie. Tijdens batchfermentatie werden ECRP koolhydraten (85%) in 24 uur iets minder gefermenteerd dan CRP koolhydraten (92%). Dit werd veroorzaakt door de lagere benutting van onoplosbare vezels in ECRP vergeleken met CRP. De lagere hoeveelheden SCFA voor ECRP komen overeen met de lagere koolhydraatbenutting van ECRP in vergelijking tot CRP.
De resultaten suggereren dat oplosbare, vlot fermenteerbare vezels zoals aanwezig in ECRP, de groei van bacteriën die goed groeien op deze vezels zodanig stimuleren dat de microbiota zich vervolgens minder goed kan aanpassen aan het benutten van onoplosbare vezels.
Om de afbraak van CRP pectine tijdens de *in vitro* batchfermentatie door humaan fecaal microbiota beter te begrijpen, werden enzymactiviteiten gevolgd in de tijd, zoals beschreven in **Hoofdstuk 5.** Enzymactiviteiten werden geïdentificeerd met behulp van PNP-glycosides en complexe polysachariden. Enzym extracten (EEs) uit CRP fermentatie vloeistoffen (verkregen na 2, 12 en 24 uur) werden gedurende 24 uur geïncubeerd met goed gekarakteriseerde polysachariden en met celwand residuen (CWRs), verkregen na sequentiële extractie uit CRP, om de werkingswijze en efficiëntie van het enzym te bepalen. De resultaten lieten een toename zien in de hoeveelheid arabinofuranosidase, β-galactosidase, endo-arabinanase, endo-galactanase en exo-polygalacturonase en pectine-afbrekende enzymen (pectinemethylesterase, endo-polygalacturonase). De hoeveelheden van deze enzymen dragen bij aan een synergie in de pectine-afbraak uit CRP tussen 12 en 24 uur fermentatie. De hypothese wordt gesteld dat de toename in afbraak in de tijd werd veroorzaakt door een toegenomen porositeit van de celwanden en een daardoor toegenomen toegankelijkheid voor de enzymen. Gecombineerd met de toegenomen hoeveelheden

pectine-afbrekende enzymen leidde dit tot een verhoogde afbreekbaarheid van resterende homogalacturonaan, arabinaan en galactaan. De hogere afbreekbaarheid van arabinaan in vergelijking met galactaan aanwezig in het netwerk werd veroorzaakt door de architectuur in CRP waarbij het arabinaan meer toegankelijk was dan galactaan.

In **Hoofdstuk 6** wordt de distributie van CWPs binnen de celwanden van CRP en ECRP voorgesteld. Verder wordt de batchfermentatie van CRP en ECRP vergeleken met TIM-2 fermentatie van CRP en ECRP. De invloed van oplosbare vezels op de fermenteerbaarheid van onoplosbare vezels wordt besproken. De enzymactiviteiten die betrokken zijn bij de fermentatie van CRP in het batch model worden bediscussieerd.

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Thank you all,

About the author

Curriculum Vitae

Uttara Ramasamy was born on the 2nd of Dec 1984 in Bangalore, India. She completed her bachelors in Microbiology from Abasaheb Garware College in Pune, Maharashtra, India. During her bachelors, she also worked part time on a project in Bioinformatics which involved the contruction of three dimensional phylogenetic trees. Towards the end of her bachelors, she undertook an internship at United Breweries in Mumbai, India. From here, she went on further, to pursue her masters in Food Technology from Wageningen University. Her master thesis dealt with kinetic modelling of enzymatic starch hydrolysis in the Food Process Engineering Group and her intership was in product development in baking in DMV-Friesland Campina. After completing her master degree, she was offered to work as a PhD student at the Labortaory of Food Chemistry under the supervision of Dr. Henk. A. Schols and Prof. Dr. Harry Gruppen. The outcome of her research during her PhD is presented in this thesis.

List of publications:

Ramasamy, U. S.; Gruppen, H.; Schols, H. A., Structural and water-holding characteristics of untreated and ensiled chicory root pulp. *Journal of Agricultural and Food Chemistry* **2013**, 61, 6077-6085.

Ramasamy U. S., Venema K., Schols H. A. and Gruppen H., The effect of soluble and insoluble fibers on the fermentation of chicory root pulp **2014**. Submitted for publication

Ramasamy U. S., Venema K., Gruppen H. and Schols H. A., The fate of chicory root pulp polysaccharides during fermentation in the TNO *In vitro* model of the colon (TIM-2) **2014**. Submitted for publication

Ramasamy U. S., Schols H. A. and Gruppen H., Characteristics of bacterial enzymes present during in vitro fermentation of chicory root pulp by human faecal microbiota **2014**. Submitted for publication

Vogt, L., **Ramasamy U.**, D. Meyer, G. Pullens, K. Venema, M. M. Faas, H. A. Schols and P. de Vos. Immune modulation by different types of β2→1-fructans is toll-like receptor dependent. *PLoS ONE*. **2013**, **8** (7): e68367.

Vogt, L., Meyer D., Pullens G., Faas M., Smelt M., Venema K., **Ramasamy U.**, Schols H. A. and Vos P. de. Immunological properties of inulin-type fructans. *Critical Reviews in Food Science and Nutrition.* **2013**. (Just-accepted).

Overview of completed training activities

Discipline specific courses

Courses

Summercourse Glycosciences * (VLAG), Wageningen, The Netherlands, 2010
Advanced Food Analysis * (VLAG), Wageningen, The Netherlands, 2010
Food and biorefinery enzymology * (VLAG), Wageningen, The Netherlands, 2010

Conferences and meetings

CCC Scientific days *^ Groningen, The Netherlands, 2009-2013
Plant cell wall conference * Porto, Portugal, 2010
Cosun Workshop ^, Royal Cosun, The Netherlands, 2010
EPNOE (European Polysaccharide Network of Excellence)*, Wageningen, The Netherlands, 2011
Plant and Sea-weed Polysaccharide Workshop*, INRA, Nantes, France 2012

General courses

Information literacy including Endnote, WGS, 2009
VLAG PhD week, 2009
Techniques for writing and presenting a scientific paper, 2010
Interpersonal communication skills, 2013
Mobilising scientific network, 2013
Career perspectives, 2013

Additional activities at the Laboratory of Food Chemistry

Preparation of PhD research proposal
PhD Trip to Switzerland and Italy, 2010
PhD Trip to Malaysia and Singapore, 2012
MSc thesis student presentations and colloquia (2009-2013)
PhD presentations (2009-2013)
Project meetings (2009-2013)

* poster presentation, ^ oral presentation

Abbreviations used:

VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Science
CCC: Carbohydrate Competence Centre
WGS: Wageningen Graduate School

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